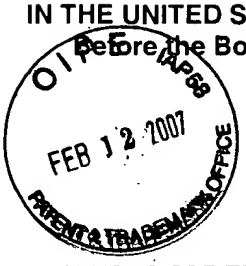
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Before the Board of Patent Appeals and Interferences



In re Patent Application of Atty Dkt. LCM-1331-352
C# M#
von BORSTEL et al TC/A.U.: 1623
Serial No. 09/930,494 Examiner: Patrick T. Lewis
Filed: August 16, 2001 Date: February 12, 2007
Title: COMPOSITIONS AND METHODS FOR TREATMENT OF MITOCHONDRIAL DISEASES

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

☐ **Correspondence Address Indication Form Attached.**

☐ **NOTICE OF APPEAL**
Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the Examiner twice/finally rejecting applicant's claim(s). $500.00 (1401)/$250.00 (2401) $ 0.00

☒ An appeal **BRIEF** is attached in the pending appeal of the above-identified application $500.00 (1402)/$250.00 (2402) $ 500.00

☐ Credit for fees paid in prior appeal without decision on merits -$ (0.00)

☐ A reply brief is attached. **(no fee)**

☐ Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s)
One Month Extension $120.00 (1251)/$60.00 (2251)
Two Month Extensions $450.00 (1252)/$225.00 (2252)
Three Month Extensions $1020.00 (1253/$510.00 (2253)
Four Month Extensions $1590.00 (1254/$795.00 (2254) $ 450.00

☐ "Small entity" statement attached.

Less month extension previously paid on -$(0.00)

TOTAL FEE ENCLOSED $ 950.00

Any future submission requiring an extension of time is hereby stated to include a petition for such time extension. The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our **Account No. 14-1140.** A duplicate copy of this sheet is attached.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
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In re Patent Application of

von BORSTEL et al — Atty. Ref.: 1331-352

Serial No. 09/930,494 — TC/A.U.: 1623

Filed: August 16, 2001 — Examiner: Patrick T. Lewis

For: COMPOSITIONS AND METHODS FOR TREATMENT OF MITOCHONDRIAL DISEASES

* *

February 12, 2007

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the Examiner.

02/13/2007 SZEWDIE1 00000085 09930494
01 FC:1402 500.00 OP

(I) REAL PARTY IN INTEREST

The real party in interest is Wellstat Therapeutics Corporation (previously known as Pro-Neuron, Inc.), a corporation of the State of California.

(II) RELATED APPEALS AND INTERFERENCES

The appellant, the undersigned, and the assignee are not aware of any related appeals, interferences, or judicial proceedings (past or present), which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(III) STATUS OF CLAIMS

Claims 1-15, 18-41 and 47-49 are pending and have been rejected. Claims 16, 17, 42-46 and 50 have been canceled. Claims 1-15, 18-41 and 47-49 are appealed.

No claims have been substantively allowed.

(IV) STATUS OF AMENDMENTS

No amendment has been filed since mailing of the final rejection on April 12, 2006.

(V) SUMMARY OF CLAIMED SUBJECT MATTER

The invention of the claims relates, in one embodiment, to a method for treating or preventing pathophysiological consequences of mitochondrial respiratory chain dysfunction in a mammal, comprising administering to the mammal in need of such treatment or prevention an effective amount of a pyrimidine nucleotide precursor (page 5, fourth complete paragraph, and page 18 onwards). In another embodiment, there is provided a method for preventing death or functional decline of post-mitotic cells in a mammal due to mitochondrial respiratory chain dysfunction, comprising administration of an effective amount of a pyrimidine nucleotide precursor (page 5, last complete paragraph, and page 18 onwards). In yet a further embodiment, there is provided a method for treating developmental delay in cognitive, motor, language, executive function, or social skills in a mammal, comprising administration of an effective amount of a pyrimidine nucleotide (page 6, first complete paragraph, and page 18 onwards).

(VI) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The grounds of rejection to be reviewed on appeal are as follows:

(1) The rejection of claims 1-15, 18-32 and 47-49 under 35 U.S.C. §112, first paragraph, on lack of enablement grounds.

(2) The rejection of claims 33-36 under 35 U.S.C. §112, first paragraph, on lack of enablement grounds.

(3) The rejection of claims 1-15, 18-32 and 37-41 under 35 U.S.C. §103(a) as unpatentable over Page et al., *Proc Natl. Acad. Sci. USA*, Vol. 94, 11601-1166 (1997) in combination with U.S. 6,316,426 to von Borstel et al.

Claims 1-15, 21, 23, 31-32, 37-41 and 47 also stand rejected as constituting obviousness-type double patenting over claims 48-59 of copending Application Serial No. 09/763,955. However, Applicants have already indicated on the record that a Terminal Disclaimer will be submitted when allowable subject matter is indicated.

(VII) ARGUMENT

I. THE 35 U.S.C. §112, FIRST PARAGRAPH, REJECTIONS

Claims 1-15, 18-32 and 47-49 stand rejected under 35 U.S.C. §112, first paragraph, for the reasons of record as set forth in the Official Action mailed July 26, 2005, namely that the specification, while enabling for the treatment of congenital mitochondrial disease, Alzheimer's Disease, Huntington's Disease, neuromuscular degenerative disease, and pathophysiological consequences of mitochondrial respiratory chain dysfunction, allegedly does not reasonably provide enablement for the prevention of congenital mitochondrial disease, Alzheimer's Disease, Huntington's Disease, neuromuscular degenerative disease, and pathophysiological consequences of mitochondrial respiratory chain dysfunction. Claims 33-36 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. Reversal of these rejections is respectfully requested.

The Final Action, at page 4, asserts that the references relied upon in support of Applicants' position are not sufficient to overcome the rejection because they were published in 2003, later than the date of filing of the present application in 2001. The Action asserts that: "Publications dated after the filing date providing information publicly first disclosed after the filing date generally cannot be used to show what was known at the time of filing." However, in this case, the references are being relied upon to show how someone of ordinary skill would have understood the term "prevention" as used in the context of the presently claimed invention as of 2001. It is Applicants' position that the understanding of one of ordinary skill would not have changed significantly if at all

over the time period from the 1998 priority date of the present application to the date of the references in 2003. The Action (page 5) counters this position by asserting that:

> "...during patent examination, the pending claims must be given their broadest reasonable interpretation consistent with the specification. This means that the words of the claim must be given their plain meaning unless applicant has provided a clear definition in the specification. In the instant case, the specification does not provide a definition of "prevention". In the absence of an express intent to impart a novel meaning to the claim terms, the words are presumed to take on the ordinary and customary meanings attributed to them by those of ordinary skill in the art."

In response, the correct connotation of "preventing" as used in the presently claimed invention, and as supported by the data presented in the specification, is the prophylactic administration of compounds of the invention which prevents progression or full manifestation of diseases related to essentially irreversible mitochondrial defects. Hereditary mitochondrial diseases typically involve genetic defects in genes coding proteins that, directly or indirectly, affect mitochondrial respiration. Diseases of acquired mitochondrial dysfunction, which may include Alzheimer's Disease, also have essentially permanent molecular defects disrupting mitochondrial respiration. The concept of "prevention" in the context of this class of diseases relates to clinical expression of symptoms stemming from the (permanent) genetic defect. The idea of prevention does not relate to preventing or reversing genetic defects but, rather, compensating for them to prevent full clinical manifestation of their disorder.

Prevention in the context of the present invention therefore applies to reducing the rate of progression of a chronic, worsening disease process compared with patients who do not receive the drug. In most cases, this will fall under the heading of "treatment" of a diagnosed disease but, in other situations, e.g. where a genetic disorder has not yet (but eventually will) cause clinical symptoms (e.g. in Huntington's Disease,

where a hereditary defect leads to adult onset of the disease after a nonsymptomatic earlier life) or Alzheimer's Disease, when a patient can be detected occur before actually meeting the diagnostic criteria for Alzheimer's Disease, the concept of prevention is medically and scientifically legitimate. Thus, in a patient with a genetic diagnosis of Huntington's Disease, administration of compounds of the invention may prevent onset of debilitating symptoms, lessen their severity once they do manifest, or slow the rate of progression.

In medical practice, these types of outcomes in progressive diseases are considered successful preventative interventions. In animal experiments, including several examples in the instant application (discussed below), prophylactic administration of triacetyluridine reduces the effects of subsequent administration of mitochondrial toxins, e.g. 3-nitropropionic acid and MPTP. Subsequent experiments with the Complex IV inhibitor sodium azide yielded similar results (see Example 12 of the present case, which also demonstrated reduction of cell loss in the nervous system). It is not that the administered drug (triacetyluridine) prevented the chemical lesion caused by the toxin (and it likewise does not reverse the genetic lesion in a patient with hereditary or congenital mitochondrial disease) but, rather, it attenuated the physiological consequences of the chemical lesion, including prevention of mortality in some cases.

The data included in the present application involves both "treatment" and "neuroprotective" effects of a pyrimidine nucleotide precursor(s), used as a therapeutic for disorders involving mitochondrial respiratory chain enzyme impairment. The experimental approach involved initiating treatment with the pyrimidine nucleotide

precursor triacetyluridine prior to the administration of the mitochondrial toxin. The complex I respiratory chain inhibitor MPTP model of Parkinson's disease (PD) (Example 7), Complex II respiratory chain inhibitor 3-nitropropionic acid model of Huntington's disease (HD) (Example 9) and the Complex IV respiratory chain inhibitor azide model of Alzheimer's disease (AD) (Example 12) included pretreatment with triacetyluridine. The treatment with triacetyluridine continued throughout the course of these examples. The sum therapeutic effect of triacetyluridine in the PD and HD and stroke models was a combined neuroprotective/cytoprotective and treatment effect. In the Complex IV respiratory chain inhibitor azide model of AD, there was a decrease in mortality due to pretreatment with triacetyluridine. If the mitochondrial impairment was not extremely severe (as was the case with the use of azide at only the 40 µg/hr dose), pretreatment/treatment with triacetyluridine was able to completely prevent mortality.

Based on the above, it would be understood by one of ordinary skill in reading the specification that "preventing" as used in the presently claimed invention means the prophylactic administration of compounds of the invention which prevents progression or full manifestation of diseases related to essentially irreversible mitochondrial defects. Just as few or no other classes of drugs used for treatment of chronic diseases prevent or reverse all symptoms completely, the standard for successful prevention in medical practice is prevention of symptoms of a disorder (especially a progressive or episodically exacerbating disorder) from being as bad as it would be without the drug. This is particularly significant for mitochondrial disorders which, as a class, often undergo exacerbations, either episodically or permanently.

The effect of an acylated ribonucleoside derivative(s) to "prevent" diseases involving mitochondrial dysfunction can be described as a "neuroprotective" and a "cytoprotective" effect (to include non-central nervous system cells). The term "neuroprotective" has been used to refer to the ability of a therapeutic method, if given prior to the initial initiation of factors that cause the disease ("pretreatment"), to reduce the severity or delay the onset and/or slow the progression of tissue damage and functional impairment (see Exhibits M, N. and O). The *in vivo* evidence described in the present case further supports the disease preventative effects achieved by the presently claimed method.

On page 5 of the Action, the conclusory statement that "there is a tremendous amount of unpredictability and uncertainty in the art" (referring to Bren, Hollander et al., Cattaneo et al.) does not provide a basis to conclude that the present specification fails to provide an enabling disclosure with regard to the prevention aspect of the presently claimed invention. The present inventors have established via the data contained in the present specification, that "preventing" as used in the claimed invention is enabled by the prophylactic administration of compounds of the invention which prevents progression or full manifestation of diseases related to essentially irreversible mitochondrial defects.

Reversal of the lack of enablement rejection of claims 1-15, 18-32 and 47-49 is respectfully requested.

The rejection of claims 33-36 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention should also be reversed for the

reasons discussed above, that the specification provides an enabling disclosure of the subject matter of these claims. With regard to the alleged lack of description of the subject matter of claims 33-36, the specification, in the last paragraph on page 5, together with the detailed description beginning on page 18, particularly the third complete paragraph on page 31 in the discussion of treatment, reversal and/or prevention of ALS, provides sufficient description of the claimed method for preventing death or functional decline of post-mitotic cells in a mammal due to mitochondrial respiratory chain dysfunction by administering an effective amount of a pyrimidine nucleotide precursor, so as to enable one of ordinary skill to carry out the method of claims 33-36.

In light of the above, it is believed that the prevention aspect of the present invention (as well as the treatment aspect) is supported by an enabling disclosure. Reversal of the outstanding 35 USC 112, first paragraph, rejections is accordingly respectfully requested.

II. THE OBVIOUSNESS REJECTION

Claims 1-15, 18-32 and 37-41 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Page et al., *Proc Natl. Acad. Sci. USA*, Vol. 94, 11601-1166 (1997) in combination with U.S. 6,316,426 to von Borstel et al. This rejection is respectfully traversed.

Page describes the use of uridine to treat patients with a rare disease associated with excess activity of the enzyme 5'-nucleotidase, an enzyme involved in degradation of nucleotides. The finding by Page that nucleotide precursors (uridine or ribose) are

clinically useful in treating a disorder in which the only known molecular deficit is an excess of an enzyme (5'-nucleotidase) involved in nucleotide degradation, would **not** have led one of ordinary skill to suspect that uridine or ribose would be useful in treating or preventing other disease conditions which might manifest similar symptoms. In scientific publications describing these patients (see attached Exhibits P and Q), there is no indication or suggestion of evidence for mitochondrial respiratory chain dysfunction as a molecular basis for symptoms of the described disease. As noted above, patients with this condition are rare, and there are no clear implications for other diseases. Based on this, the Page disclosure clearly does not render the presently claimed invention obvious.

The above-noted deficiencies of Page are not cured by the '426 U.S. patent to von Borstel. The '426 U.S. patent discloses that acylated ribonucleoside derivatives are effective in treating a number of disorders that involve functional impairments in tissue and organ systems involving metabolic deficiencies. However, these metabolic deficiencies in the '426 US patent are not asserted to be due to respiratory chain enzyme impairment. In view of this, one of ordinary skill would not have been motivated to combine the Page and von Borstel disclosures in the context of the presently claimed invention. Absent any such motivation, no *prima facie* case of obviousness is established in this case.

The Final Action asserts (pages 7 and 8) that "Developmental delay, seizures, ataxia, recurrent infections, severe language deficit, and an unusual behavioral phenotype characterized by hyperactivity, short attention span, and poor social

interaction are 'pathophysiological consequences of mitochondrial respiratory chain dysfunction' ". This assertion is respectfully traversed.

It is not true that all of the conditions recited in the Action are necessarily 'pathophysiological consequences of mitochondrial respiratory chain dysfunction'. As it is well recognized by person of ordinary skill that unrelated diseases can have overlapping symptoms, it is equally well recognized that the effectiveness of a particular drug in treating a symptom in one disorder does not necessarily, or even generally, imply that the drug will be useful in treating other diseases with similar symptoms. For example, epilepsy or related seizure disorders may be caused by tumors, poisons, mitochondrial defects, or simply self-amplifying circuits of neural activity without other organic defects causing the seizures. Seizure episodes in a susceptible person can be triggered by progesterone deficits, e.g. associated with the menstrual cycle. Although the clinical symptoms – seizures – may look similar, the treatments will vary according to the underlying problem.

Valproate (Depakote) is a widely-used anti-seizure medication, but it can actually exacerbate seizures (and other manifestations of mitochondrial disease) caused by mitochondrial deficits, due to its inhibitory effect on mitochondrial respiration. For someone with seizures triggered by a progesterone deficit, progesterone or an analog thereof is more appropriate than increased doses of other anti-seizure medications, which have debilitating side effects at higher doses. Some seizure disorders associated with foci of hyperexcitable neurons are best treated with electrodes inserted into the brain, which would be inappropriate for seizures caused by metabolic deficits. As evidence of this, attention is directed to Exhibits A-D, which are briefly discussed below.

Lam et al (Eur J Pediatr. 1997 Jul;156(7):562-4. Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) triggered by valproate therapy. Lam CW, Lau CH, Williams JC, Chan YW, Wong LJ. Department of Pathology, Princess Margaret Hospital, Lai Chi Kok, Hong Kong) (Exhibit A) have reported that:

> "...valproate should not be given to patients suspected of having mitochondrial diseases. In addition, for patients whose seizures worsen with valproate therapy, an inborn error of mitochondrial metabolism should be suspected. The underlying mitochondrial DNA defects should be sought for family screening and genetic counselling."

Likewise, Krahenbuhl et al (Liver. 2000 Jul;20(4):346-8; Mitochondrial diseases represent a risk factor for valproate-induced fulminant liver failure. Krahenbuhl S, Brandner S, Kleinle S, Liechti S, Straumann D. Department of Clinical Pharmacology, University of Berne, Switzerland) (Exhibit B) have reported that "Mitochondrial diseases should therefore be considered as a risk factor for valproate-induced liver failure and be excluded before treatment with valproate."

Another example of a condition which can arise from different causes is arthritis. Pain in the joints can be caused by autoimmune attack (rheumatoid arthritis, psoriatic arthritis, or lupus-associated), osteoarthritis, infections, e.g. lime disease, gout, deposition of antibody complexes, etc. All of these disorders may present with joint pain as a predominant symptom, but the appropriate treatments are very different for each of these different diseases that underlie similar symptoms, e.g. anti-TNF therapies for rheumatoid arthritis, B-Cell suppressors for Lupus, nonsteroidal anti-inflammatory drugs for osteoarthritis, antibiotics for Lyme disease, allopurinol for gout. Attention in this regard is directed to Ritchie et al., "Diagnostic Approach to Polyarticular Joint Pain",

American Family Physician, 68, 6, 1151-1160 (2003) (Exhibit C), which states (in the Abstract) that "Identifying the cause of polyarticular joint pain can be difficult because of the extensive differential diagnosis." As a consequence, "...family physicians need to keep the diagnosis open in evaluating patients who present with pain in multiple joints." (page 1151, left hand column).

Many other examples are possible in which symptoms themselves provide inadequate information for determining their cause and appropriate treatment. Developmental delays may arise from a variety of underlying causes, including metabolic defects such as phenylketonuria, lead or mercury poisoning, epilepsy, or a variety of genetic defects. A diet low in phenylalanine helps patients with phenylketonuria (in which an enzyme deficiency prevents phenylalanine metabolism), but is useless in other conditions involving developmental delay or seizures. Lead and mercury poisoning can perhaps helped by administration of chelating agents which are useless in diseases not caused by heavy metals. Antiepileptic drugs like valproate or lamictal can help developmental delays secondary to disruptions in brain function caused by seizures, but may be detrimental in disorders not caused by seizures.

The relationship between the molecular anomaly, 5'nucleotidase excess, and symptoms in the children described by Page et al. is not clear. As the authors point out, the disorder is not associated with actual uridine nucleotide deficits (and the symptoms do not match those of the only known pyrimidine deficit disorder, Orotic Aciduria). Uridine and related pyrimidine compounds were initially tested in these patients because the first one identified presented with megaloblastic anemia (a primary symptom of orotic aciduria), which was later attributed to her anti-seizure medication.

The finding that uridine was helpful was actually fortuitous and does not provide a basis for asserting that uridine would be helpful in similar symptoms or symptom complexes associated with other diseases.

In addition, the cited Page et al paper is not the first publication of the use of uridine to treat 5'-nucleotidase excess. This was published earlier in Page, et al., "A Syndrome of Megaloblastic Anemia, Immunodeficiency, and Excessive Nucleotide Degradation," in Purine and Pyrimidine Metabolism in Man VII, Part B, Harkness, et al. eds (1991) pp. 345-348 (Exhibit D). The fact that between 1991 and the subject invention no one used uridine compounds to treat pathophysiological consequences of mitochondrial respiratory chain dysfunction is further evidence of its nonobviousness.

Prior to the effective filing date of the subject application, a number of diseases were known to be mitochondrial in origin. Yet they were not treated with pyrimidine nucleotide precursors. This observation refutes the Office's position that it would have been obvious to treat any and all mitochondrial diseases using pyrimidine nucleotide precursors. As evidence of this, attention is directed to Exhibits E-L, which show that, while various types of therapies for mitochondrial disorders have been suggested (including, for example, administration of vitamins, cofactors, antioxidants, nutrients, buffers for intracellular ATP, free radical scavengers, NOS inhibitors), the method of administering a pyrimidine nucleotide precursor according to the present invention has not been suggested.

In light of the above, it is believed that a *prima facie* case of obviousness has not been generated in this case. Reversal of the obviousness rejection is respectfully requested.

III. OBVIOUSNESS-TYPE DOUBLE PATENTING

Claims 1-15, 21, 23, 31-32, 37-41 and 47 stand provisionally rejected on obviousness-type double patenting grounds as allegedly unpatentable over claims 48-59 of copending Application Serial No. 09/763,955. Applicants will consider filing a Terminal Disclaimer when otherwise allowable subject matter is indicated.

CONCLUSION

In conclusion it is believed that the outstanding rejections should be reversed. Such action is respectfully requested.

Respectfully submitted,

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(VIII) CLAIMS APPENDIX

1. A method for treating or preventing pathophysiological consequences of mitochondrial respiratory chain dysfunction in a mammal comprising administering to said mammal in need of such treatment or prevention an effective amount of a pyrimidine nucleotide precursor.

2. A method as in claim 1 wherein said respiratory chain dysfunction is caused by a mutation, deletion, or rearrangement of mitochondrial DNA.

3. A method as in claim 1 wherein said respiratory chain dysfunction is caused by defective nuclear-encoded protein components of the mitochondrial respiratory chain.

4. A method as in claim 1 wherein said respiratory chain dysfunction is caused by aging.

5. A method as in claim 1 wherein said respiratory chain dysfunction is caused by administration of cytotoxic cancer chemotherapy agents to said mammal.

6. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex I activity.

7. A method as in claim 1 wherein said respiratory chain dysfunction is a

deficit in mitochondrial Complex II activity.

8. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex III activity.

9. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex IV activity.

10. A method as in claim 1 wherein said respiratory chain dysfunction is a deficit in mitochondrial Complex V activity.

11. A method as in claim 1 wherein said pyrimidine nucleotide precursor is selected from the group consisting of uridine, cytidine, an acyl derivative of uridine, an acyl derivative of cytidine, orotic acid, an alcohol ester of orotic acid, or a pharmaceutically acceptable salt thereof.

12. A method as in claim 11 wherein said pyrimidine nucleotide precursor is an acyl derivative of cytidine.

13. A method as in claim 11 wherein said pyrimidine nucleotide precursor is an acyl derivative of uridine.

14. A method as in claim 11 wherein said acyl derivative of uridine is 2',3',5'-

tri-O-acetyluridine.

15. A method as in claim 11 wherein said acyl derivative of uridine is 2',3',5'-tri-*O*-pyruvyluridine.

18. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered orally.

19. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered in a dose of 10 to 1000 milligrams per kilogram of bodyweight per day.

20. A method as in claim 11 wherein said pyrimidine nucleotide precursor is administered in a dose of 100 to 300 milligrams per kilogram of bodyweight per day.

21. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a congenital mitochondrial disease.

22. A method as in claim 21 wherein said congenital mitochondrial disease is selected from the group consisting of Mitochondrial Encephalomyopathy, Lactic Acidemia, and stroke like episodes; Lerber's Hereditary Optic Neuropathy; Myclonic Epilepsy and "Ragged Red" (muscle) Fibers; Mitochondrial neurogastrointestinal encephalomyopathy; Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa; Progressive External Ophthalmoplegia; Leigh's Disease; and Kearns-Sayres

Syndrome.

23. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a neurodegenerative disease.

24. A method as in claim 23 wherein said neurodegenerative disorder is Alzheimer's Disease.

25. A method as in claim 23 wherein said neurodegenerative disorder is Parkinson's disease.

26. A method as in claim 23 wherein said neurodegenerative disorder is Huntington's Disease.

27. A method as in claim 23 wherein said neurodegenerative disorder is age-related decline in cognitive function.

28. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is a neuromuscular degenerative disease.

29. A method as in claim 28 wherein said neuromuscular degenerative disease is selected from the group consisting of muscular dystrophy, myotonic dystrophy, chronic fatigue syndrome, and Friedreich's Ataxia.

30. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is developmental delay in cognitive, motor, language, executive function, or social skills.

31. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is selected from the group consisting of epilepsy, peripheral -neuropathy, optic neuropathy, autonomic neuropathy, neurogenic bowel dysfunction, sensorineural deafness, neurogenic bladder dysfunction, migraine, and ataxia.

32. A method as in claim 1 wherein said pathophysiological consequence of mitochondrial respiratory chain dysfunction is selected from the group consisting of renal tubular acidosis, dilating cardiomyopathy, steatohepatitis, hepatic failure, and lactic acidemia.

33. A method for preventing death or functional decline of post-mitotic cells in a mammal due to mitochondrial respiratory chain dysfunction comprising administration of an effective amount of a pyrimidine nucleotide precursor.

34. A method as in claim 33 wherein said post-mitotic cells are neurons.

35. A method as in claim 33 wherein said post-mitotic cells are skeletal

muscle cells.

36. A method as in claim 33 wherein said post-mitotic cells are cardiomyocytes.

37. A method for treating developmental delay in cognitive, motor, language, executive function, or social skills in a mammal comprising administration of an effective amount of a pyrimidine nucleotide.

38. A method as in claim 37 wherein said developmental delay is pervasive developmental delay or pervasive developmental delay – not otherwise specified.

39. A method as in claim 37 wherein said developmental delay is Attention Deficit/Hyperactivity Disorder.

40. A method as in claim 37 wherein said developmental delay is Rett's Syndrome.

41. A method as in claim 37 wherein said developmental delay is autism.

47. A method as in Claim 1 further comprising administering pyruvic acid, a pharmaceutically acceptable salt thereof, or a pyruvic acid ester.

48. A method as in claim 1 further comprising administering to the mammal an amount of creatine such that the combined amount of creatine and the pyrimidine nucleotide is effective to treat said consequences of mitochondrial respiratory chain dysfunction.

49. A method as in claim 48 wherein said pyrimidine nucleotide is 2',3',5'-tri-O-acetyluridine.

(IX) EVIDENCE APPENDIX

Exhibit A: Lam et al (Eur J Pediatr. 1997 Jul;156(7):562-4 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit B: Krahenbuhl et al (Liver. 2000 Jul;20(4):346-8 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit C: Ritchie et al., "Diagnostic Approach to Polyarticular Joint Pain", *American Family Physician*, 68, 6, 1151-1160 (2003) (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit D: Page, et al., "A Syndrome of Megaloblastic Anemia, Immunodeficiency, and Excessive Nucleotide Degradation," in Purine and Pyrimidine Metabolism in Man VII, Part B, Harkness, et al. eds (1991) pp. 345-348 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit E: DiMauro, et al, "Mitochondrial encephalomyopathies: where next?", Revista de Neurologia (1999) 28(2):164-168 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit F: Luft, "Review: The development of mitochondrial medicine", Proc. Natl. Acad. Sci. USA (September 1994) 91: 8731-8738 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit G: Beal, "Mitochondrial dysfunction in neurodegenerative diseases", Biochimica et Biophysica Acta (1998) 1366: 211-223 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit H: Blass, "Brain metabolism and brain disease: is metabolic deficiency the proximate cause of Alzheimer dementia", J. Neurosc. Res. (2001) 66: 851-856 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit I: Bowling, et al., "Minireview: Bioenergetic and Oxidative stress in neurodegenerative diseases", Life Sciences (1995) 56(14): 1151-1171 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit J: Beal, "Mitochondria, free radicals, and neurodegeneration", Current Opinion Neurobiol. (1996) 6: 661-666 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit K: Browne, et al, "Oxidative damage and mitochondrial dysfunction in neurodegenerative diseases", Biochem. Soc. Trans. (1994) 22: 1002-1006 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit L: Schulz, et al., "Mitochondrial dysfunction in movement disorders", Current Opinion in Neurology (1994) 7:333-339 (submitted with the Response dated January 26, 2006 and entered per the Action mailed April 12, 2006).

Exhibit M: Ferrante, et al., "Neuroprotective Effects of Creatine in a Transgenic Mouse Model of Huntington's Disease"; *The Journal of Neuroscience*; 20(12), pp 4389-4397, June 15, 2000 (submitted with the Response dated April 21, 2004 and entered per the Action mailed November 3, 2004).

Exhibit N: Du et al., "Minocycline Prevents Nigrostriatal Dopaminergic Neurodegeneration in the MPTP Model of Parkinson's Disease"; *PNAS*, Vol. 98, No. 25; pp. 14469-14674 (December 4, 2001) (submitted with the Response dated April 21, 2004 and entered per the Action mailed November 3, 2004).

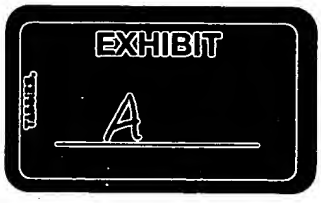
Exhibit O: Ravina et al., "Neuroprotective Agents for Clinical Trials in Parkinson's Disease"; *American Academy of Neurology*; 60, pp. 1234-1240 (2003) (submitted with the Response dated April 21, 2004 and entered per the Action mailed November 3, 2004).

Exhibit P: Page et al., Adv. Exp. Med. Biol. 1998; 431:789-92 (submitted with the Response dated April 21, 2004 and entered per the Action mailed November 3, 2004).

Exhibit Q: Page et al., Adv. Exp. Med. Biol. 1991; 309B:345-8 (submitted with the Response dated April 21, 2004 and entered per the Action mailed November 3, 2004).

(X) RELATED PROCEEDINGS APPENDIX

None.



Eur J Pediatr. 1997 Jul;156(7):562-4.

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) triggered by valproate therapy.

Lam CW, Lau CH, Williams JC, Chan YW, Wong LJ.

Department of Pathology, Princess Margaret Hospital, Lai Chi Kok, Hong Kong.

We report in this study a patient who developed repeated convulsions as a result of valproate therapy. MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) was subsequently diagnosed and a nucleotide 3243 A-->G mutation was detected in the mitochondrial DNA. This mutation predisposes the patient to the detrimental effects of valproate on oxidative phosphorylation. CONCLUSION: We support the suggestion of Ponchaut et al. [14] that valproate should not be given to patients suspected of having mitochondrial diseases. In addition, for patients whose seizures worsen with valproate therapy, an inborn error of mitochondrial metabolism should be suspected. The underlying mitochondrial DNA defects should be sought for family screening and genetic counselling.

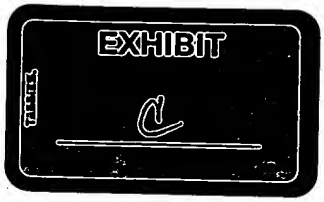
Liver. 2000 Jul;20(4):346-8.

Mitochondrial diseases represent a risk factor for valproate-induced fulminant liver failure.

Krahenbuhl S, Brandner S, Kleinle S, Liechti S, Straumann D.

Department of Clinical Pharmacology, University of Berne, Switzerland.

We report on 3 siblings (2 females and 1 male) with chronic progressive external ophthalmoplegia (CPEO), compatible with inherited mitochondrial cytopathy. The younger of the two sisters died at the age of 37 due to progressive respiratory failure. The older one presented with a status epilepticus at the age of 39 and was treated with valproate. Five months after the start of treatment, she developed fulminant liver failure and died. The brother has suffered from CPEO since early childhood but has had so far no other symptoms of a mitochondrial disease. A muscle biopsy from the younger sister revealed ragged-red fibers and decreased activities of complex I and IV of the respiratory chain but no pathogenic mutations in the mitochondrial tRNA genes or in several locations in the coding region of the mitochondrial genome. In the older sister's liver (obtained post-mortem), mitochondrial DNA was fragmented and could not be investigated. The clinical presentation and the biochemical findings suggest that all 3 siblings suffered from a mitochondrial cytopathy. Since mitochondrial cytopathies and valproate-induced fulminant liver failure are both rare events, an association between them is likely. Mitochondrial diseases should therefore be considered as a risk factor for valproate-induced liver failure and be excluded before treatment with valproate.



Diagnostic Approach to Polyarticular Joint Pain

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Identifying the cause of polyarticular joint pain can be difficult because of the extensive differential diagnosis. A thorough history and a complete physical examination are essential. Six clinical factors are helpful in narrowing the possible causes: disease chronology, inflammation, distribution, extra-articular manifestations, disease course, and patient demographics. Patients with an inflammatory arthritis are more likely to have palpable synovitis and morning stiffness; if the condition is severe, they may have fever, weight loss, and fatigue. Viral infections, crystal-induced arthritis, and serum sickness reactions are common causes of acute, self-limited polyarthritis. Because chronic arthritides may present abruptly, they need to be considered in patients who present with acute polyarticular joint pain. Joint palpation can help to distinguish inflammatory synovitis from the bony hypertrophy and crepitus that typically occur with osteoarthritis. Extra-articular manifestations of rheumatologic disease may be helpful in arriving at a more specific diagnosis. Many classic rheumatologic laboratory tests are nonspecific. A complete blood count, urinalysis, and a metabolic panel may provide more useful diagnostic clues. Plain-film radiographs may demonstrate classic findings of specific rheumatologic diseases; however, radiographs can be normal or only show nonspecific changes early in the disease process.
(Am Fam Physician 2003;68:1151-60.

Members of various family practice departments develop articles for "Problem-Oriented Diagnosis." This is one in a series from the Department of Family and Community Medicine at Southern Illinois University School of Medicine, Springfield. Guest editor of the series is Robert M. Wesley, M.A.

Polyarticular joint pain (i.e., pain in more than four joints) poses a diagnostic challenge because of the extensive differential diagnosis[1] *(Table 1)*. Consequently, family physicians need to keep the diagnosis open in evaluating patients who present with pain in multiple joints. For instance, a 50-year-old woman with symmetric, progressive polyarticular joint swelling and prolonged morning stiffness would seem to have rheumatoid arthritis. However, this patient might develop a malar rash and oral ulcers, which would change the diagnosis to systemic lupus erythematosus. Alternatively, the patient might develop thickening of the skin, which would suggest the diagnosis of scleroderma. Thus, a series of visits over time may be necessary to arrive at a specific diagnosis in many patients with polyarticular joint pain. In some patients, it may not be possible to establish a definitive diagnosis.

Because many rheumatologic laboratory tests lack the desired specificity, results should be interpreted in the clinical context and with caution. Tests with low specificity, such as those in arthritis panels, are frequently positive in the general population. Thus, these tests may be misleading.[2] Furthermore, use of tests with low specificity may increase unnecessary testing and attendant costs, result in inappropriate treatment, and have a negative psychologic impact on patients.[3]

In the absence of definitive rheumatologic laboratory tests, the history and physical examination are key to the early diagnosis and treatment of conditions that cause polyarticular joint pain. Indeed, the differential diagnosis can be narrowed through investigation of six clinical factors: disease chronology, inflammation, distribution, extra-articular manifestations, disease course, and patient demographics *(Table 2)*. More common causes of polyarticular joint pain should be considered first.

See page 1039 for definitions of strength-of-evidence levels.

Disease Chronology

Acute polyarticular joint pain (i.e., pain that has been present for less than six weeks) may be the sign of a self-limited disorder or a harbinger of chronic disease. Although chronic polyarticular arthritides more often develop insidiously, they can present abruptly. Thus, chronic conditions such as rheumatoid arthritis and systemic lupus erythematosus should be considered, at least initially, in patients who present with acute polyarticular joint pain *(Table 3)*.[4-7] To avoid treating a self-limited

TABLE 1

Differential Diagnosis of Polyarticular Joint Pain

Viral infection: human parvovirus (especially B19), enterovirus, adenovirus, Epstein-Barr, coxsackievirus (A9, B2, B3, B4, B6), cytomegalovirus, rubella, mumps, hepatitis B, varicella-zoster virus (human herpes virus 3), human immunodeficiency virus

Indirect bacterial infection (reactive arthritis): *Neisseria gonorrhoeae* (gonorrhea), bacterial endocarditis, Campylobacter species, Chlamydia species, Salmonella species, Shigella species, Yersinia species, *Tropheryma whippelii* (Whipple's disease), group A streptococci (rheumatic fever)

Direct bacterial infection: *N. gonorrhoeae, Staphylococcus aureus,* gram-negative bacilli, bacterial endocarditis

Other infections: *Borrelia burgdorferi* (Lyme disease), *Mycobacterium tuberculosis* (tuberculosis), fungi

Crystal-induced synovitis: gout, pseudogout (calcium pyrophosphate deposition disease), hydroxyapatite

Systemic rheumatic disease: rheumatoid arthritis, systemic lupus erythematosus, polymyositis/dermatomyositis, juvenile rheumatoid arthritis, scleroderma, Sjögren's syndrome, Behçet's syndrome, polymyalgia rheumatica

Systemic vasculitis disease: Schönlein-Henoch purpura, hypersensitivity vasculitis, polyarteritis nodosa, Wegener's granulomatosis, giant cell arteritis

Spondyloarthropathies: ankylosing spondylitis, psoriatic arthritis, inflammatory bowel disease, reactive arthritis (Reiter's syndrome)

Endocrine disorders: hyperparathyroidism, hyperthyroidism, hypothyroidism

Malignancy: metastatic cancer, multiple myeloma

Others: osteoarthritis, hypermobility syndromes, sarcoidosis, fibromyalgia, osteomalacia, Sweet's syndrome, serum sickness

TABLE 2

Common Causes of Polyarticular Joint Pain

			Distribution				
Disease	*Chronology*	*Inflammation*	*Pattern*	*Symmetry*	*Axial involvement*	*Extra-articular manifestations*	*Female-to-male ratio*
Human parvovirus B19 infection	Acute	Yes	Small joints	Yes	No	Lacy rash, malar rash	3:1 to 4:1
Rheumatoid arthritis	Chronic	Yes	Small and large joints	Yes	Cervical	Subcutaneous nodules, carpal tunnel syndrome	3:1 to 4:1
Systemic lupus erythematosus	Chronic	Yes	Small joints	Yes	No	Malar rash, oral ulcers, serositis (pleuritis or pericarditis)	9:1
Osteoarthritis	Chronic	No	Lower extremity joints, proximal and distal interphalangeal joints, first carpometacarpal joint	Yes/No	Cervical and lumbar	None	1:1 to 2:1
Fibromyalgia	Chronic	No	Diffuse	Yes	Yes	Myalgias, tender points, irritable bowel syndrome	9:1
Ankylosing spondylitis	Chronic	Yes	Large joints	Yes	Yes	Iritis, tendonitis, aortic insufficiency	1:1 to 1:5
Psoriatic arthritis	Chronic	Yes	Large and small joints	Yes/No	Yes/No	Psoriasis, dactylitis ("sausage digits"), tendonitis, onychodystrophy	1:1

TABLE 3

Diagnostic Criteria for Rheumatoid Arthritis, Systemic Lupus Erythematosus, and Fibromyalgia

The rightsholder did not grant rights to reproduce this item in electronic media. For the missing item, see the original print version of this publication.

Palpation of joint capsules can help to distinguish inflammatory synovitis from the noninflammatory bony hypertrophy that often indicates osteoarthritis.

disorder with potentially toxic disease-modifying agents, synovitis should be present for six weeks before rheumatoid arthritis is diagnosed.[4] [Evidence level C, consensus opinion]

Viruses (e.g., human parvovirus B19, hepatitis viruses), crystals, and serum sickness reactions are known causes of acute, self-limited polyarthritis. The specific cause of virus-induced arthritis is not always investigated; thus, the prevalence of viruses as the etiology of arthritis may be underestimated.[8]

Except for *Neisseria gonorrhoeae,* direct bacterial infections in joints seldom cause polyarthritis.[9] Although typically oligoarticular, extra-articular bacterial infections may induce acute arthritis. Classic reactive arthritis, for example, is associated with enteric infections (Salmonella, Shigella, Campylobacter, or Yersinia species) and urogenital infections *(Chlamydia trachomatis).*

Early gout usually affects only one joint. However, this disease also should be considered in patients with acute polyarticular arthritis, particularly older women who are taking diuretics and have hypertrophy and degenerative changes of the distal interphalangeal (DIP) joints (Heberden's nodes) and proximal interphalangeal (PIP) joints (Bouchard's nodes).[10]

Inflammation

Arthritis is joint pain with inflammation, whereas arthralgia is joint pain without inflammation. The patient who presents with psoriasis and knee pain in the absence of inflammation may have the dual diagnosis of psoriasis and osteoarthritis. However, the patient who also has inflammation probably has psoriatic arthritis, which may require more aggressive therapy. Inflammatory arthritides include infectious arthritis, gout, rheumatoid arthritis, systemic lupus erythematosus, and reactive arthritis.

Cardinal signs of inflammation include erythema, warmth, pain, and swelling. Patients with severe joint inflammation or systemic disease also may present with fatigue, weight loss, or fever.[8] Morning stiffness lasting longer than one hour suggests underlying inflammation.[1] The duration of morning stiffness provides a useful guide to the extent of inflammation. For instance, morning stiffness associated with rheumatoid arthritis may last for hours.[11,12]

Palpation of multiple joint capsules is important to look for soft tissue swelling and effusions that result in edema and influx of inflammatory cells into and around the synovium. Soft tissue swelling should be distinguished from noninflammatory bony hypertrophy, such as Heberden's and Bouchard's nodes, which often indicate osteoarthritis *(Figure 1).* Crepitus indicates the pres-

FIGURE 1:

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ence of irregularities of the articular cartilage, which most commonly are associated with osteoarthritis, injury, or previous inflammation.

Because findings can be subtle, it is important to palpate each hand joint. Although palpation often can identify synovitis, it may not detect inflammation of more proximal joints in, for example, elderly patients with polymyalgia rheumatica.[13]

Morning stiffness and a history of swelling suggest an inflammatory process but also are characteristic of fibromyalgia, a noninflammatory condition *(Table 3)*.[4-7] Typically, patients with fibromyalgia have a subjective sense of swelling but no objective signs of synovitis. Fibromyalgia is suggested by the presence of polyarticular joint pain without synovitis, along with myalgias and tender points.[14]

Distribution

PATTERN

The pattern of joint involvement provides diagnostic clues. For instance, osteoarthritis of the hand usually involves the DIP and PIP joints, but not the metacarpophalangeal (MCP) joints.[15] Alternatively, rheumatoid arthritis of the hand most often involves the PIP and MCP joints, but not the DIP joints.[4,15] Psoriatic arthritis, crystal-induced arthritis, and sarcoidosis may affect all of these joints. Hand synovitis is distinctly unusual in chronic Lyme disease.[16]

Spondyloarthropathies typically involve the larger joints of the lower extremities. Osteoarthritis tends to spare wrists, elbows, and ankles, unless there is a history of trauma, inflammation, or a metabolic disorder such as hemochromatosis.

Depending on the underlying cause, the pattern of arthritis may change over time. For example, the acute stage of Lyme disease may include polyarticular arthralgias, whereas the chronic phase may include oligoarthritis, primarily in the knees.[17]

SYMMETRY

Joint involvement tends to be symmetric in systemic diseases such as rheumatoid arthritis, systemic lupus erythematosus, polymyalgia rheumatica, viral arthritides, and serum sickness reactions. Of eight variables examined in one study,[18] symmetric pain was the most potent discriminating feature for rheumatoid arthritis. Psoriatic arthritis, reactive arthritis, and gout are more likely to present with asymmetric peripheral involvement.[1,19,20]

Joint involvement tends to be symmetric in systemic diseases such as rheumatoid arthritis, systemic lupus erythematosus, and viral arthritides.

AXIAL INVOLVEMENT

Axial pain may be a helpful indicator in the evaluation of peripheral joint pain. In addition to peripheral joints, osteoarthritis may involve the lower back, the neck, or both. In contrast, rheumatoid arthritis is seldom an explanation for low back pain.

A young adult who presents with peripheral arthritis accompanied by the insidious onset of chronic low back pain and prolonged morning stiffness that improves with exercise probably has one of the spondyloarthropathies, such as ankylosing spondylitis, psoriatic arthritis, inflammatory bowel disease–associated arthropathy, or reactive arthritis.[21] Another common manifestation of spondyloarthropathies is enthesitis (inflammation of the muscular or tendinous insertions),[22] such as Achilles tendonitis or plantar fasciitis.[23] Dactylitis (inflammation of the finger or toe) is another classic sign of spondyloarthropathies; this condition, often referred to as "sausage digits," is caused by a combination of synovitis and enthesitis[22] *(Figure 2)*.

FIGURE 2.

TABLE 4

Selected Extra-Articular Manifestations Associated with Conditions That Result in Polyarticular Joint Pain*

Physical finding	*Diagnoses to consider*
Skin and mucous membranes	
Rash	
Erythema infectiosum	
Reticulated (lacy) rash	Human parvovirus B19 infection
Facial exanthem (slapped cheek)	Human parvovirus B19 infection
Malar rash	SLE, human parvovirus B19 infection, Lyme disease, rosacea, seborrhea, dermatomyositis
Plaques (scalp, navel, gluteal cleft)	Psoriasis
Heliotrope	Dermatomyositis
Erythema chronicum migrans	Lyme disease
Erythema marginatum rheumaticum	Rheumatic fever
Erythema nodosum	Sarcoidosis, Crohn's disease
Pyoderma gangrenosum	IBD, RA, SLE, anklyosing spondylitis, sarcoidosis, Wegener's granulomatosis
Palpable purpura	Hypersensitivity vasculitis, Schönlein-Henoch purpura, PAN
Livedo reticularis	Antiphospholipid-antibody syndrome, vasculitis, cholesterol emboli
Lesions	
Keratoderma blennorrhagicum	Reactive arthritis, psoriatic arthritis
Discoid skin lesions	Discoid lupus erythematosus, SLE, sarcoidosis
Gottron's papules or plaques	Dermatomyositis
Vesicopustule on erythematous base	Gonococcal arthritis
Eyes	
Iritis or uveitis	Spondyloarthropathies, sarcoidosis, Wegener's granulomatosis
Conjunctivitis	Spondyloarthropathies, SLE, Wegener's granulomatosis
Cytoid bodies (retinal exudates)	SLE
Scleritis	RA, relapsing polychondritis
Ischemic optic neuritis	Giant cell arteritis, Wegener's granulomatosis
Ears, nose, and throat	
Oral ulcers	SLE, Behçet's syndrome, reactive arthritis, Wegener's granulomatosis
Parotid enlargement	Sjögren's syndrome, sarcoidosis
Macroglossia	Amyloidosis
Scalp tenderness	Giant cell arteritis
Bloody or severe sinusitis	Wegener's granulomatosis
Inflammation of ear lobe	Relapsing polychondritis
Nails	
Onycholysis	Psoriatic arthritis, hyperthyroidism
Pitting	Psoriatic arthritis
Clubbing	IBD, Whipple's disease, hyperthyroidism
Nodules	RA, gout, Whipple's disease, rheumatic fever, amyloidosis, sarcoidosis
Tophi	Gout
Jaundice	Hepatitis, hemochromatosis
Hyperpigmentation	Whipple's disease, hemochromatosis
Telangiectasia	Scleroderma
Thickened skin	Scleroderma, amyloidosis, eosinophilic fasciitis
Hair thinning	Hypothyroidism, SLE
Musculoskeletal system	
Tender points	Fibromyalgia
Heberden's nodes (DIP joints), Bouchard's nodes (PIP joints)	Osteoarthritis
Boutonnière and swan-neck deformities	RA, SLE, Ehlers-Danlos syndrome
Dactylitis ("sausage digits")	Spondyloarthropathies
Bursitis and enthesitis	Spondyloarthropathies
Constitutional conditions	
Fever	Bacterial or viral infection, Still's disease, subacute bacterial endocarditis, neoplasm
Bradycardia	Hypothyroidism
Cardiovascular system	
Mitral regurgitation and stenosis	Rheumatic fever
Aortic regurgitation	Ankylosing spondylitis, rheumatic fever, relapsing polychondritis, reactive arthritis, Marfan syndrome, Takayasu's arteritis
Cardiomyopathies	Viral infection, amyloidosis, sarcoidosis, SLE, polymyositis
New murmur, fever	Bacterial endocarditis, rheumatic fever
Diminished peripheral pulses	Giant cell arteritis, Takayasu's arteritis
Gastrointestinal system	
Splenomegaly	Felty's syndrome, tumor-associated arthritis
Hepatomegaly	Whipple's disease, hemochromatosis, amyloidosis, Wilson's disease
Positive fecal occult blood test	IBD
Genitourinary system	
Prostatitis	Reactive arthritis, ankylosing spondylitis
Urethritis or cervicitis	Reactive arthritis, gonococcal arthritis
Scrotal or vulvar ulcers	Behçet's syndrome
Hypogonadism	Hemochromatosis
Balanitis circinata	Reactive arthritis
Neurologic system	
Entrapment neuropathies	RA, hypothyroidism, hyperparathyroidism
Facial palsy	Lyme disease
Peripheral neuropathy	SLE, amyloidosis
Chorea	Antiphospholipid-antibody syndrome, SLE, rheumatic fever
Mononeuritis multiplex	RA, SLE, Lyme disease, vasculitis (e.g., PAN)
Seizures	SLE
Lymphadenopathy	Tumor-associated arthritis, SLE

SLE = systemic lupus erythematosus; IBD = inflammatory bowel disease; RA = rheumatoid arthritis; PAN = polyarteritis nodosa; DIP = distal interphalangeal; PIP = proximal interphalangeal.

**—The clues listed in this table are not, in themselves, diagnostic or complete; they are presented for illustrative purposes only.*

FIGURE 3.

A complete blood count, urinalysis, and a metabolic panel may provide more useful diagnostic clues than classic rheumatologic laboratory tests.

Extra-Articular Manifestations

Extra-articular manifestations may provide clues to the presence of some rheumatologic diseases but, of themselves, are not diagnostic *(Table 4)*. For instance, extra-articular signs and symptoms can point to the likely reason for swollen PIP joints: a malar rash and oral ulcers indicate probable systemic lupus erythematosus *(Figure 3)*; proximal muscle weakness suggests polymyositis; and psoriatic skin and nail lesions raise the possibility of psoriatic arthritis.[24,25]

Similarly, in a patient with knee arthritis, the presence of conjunctivitis, oral ulcers, vesicopustules on the soles, or recent diarrhea may indicate reactive arthritis.[21,26] A history of erythema chronicum migrans and Bell's palsy points to the diagnosis of Lyme disease.[27] As a final example, a health care worker who presents with fever, a lacy rash, and symmetric joint pain (especially in the hands) may have erythema infectiosum caused by human parvovirus B19 infection.[28-30]

Disease Course

INTERMITTENT ARTHRITIS

When symptoms are present for a limited period (usually a few days to a month) and resolve completely before presenting again, crystal-induced arthritis (e.g., gout, pseudogout) is the likely diagnosis. Arthrocentesis should be considered during a symptomatic flare.[10,19,27,31] If synovial fluid analysis fails to identify crystals, palindromic rheumatism should be considered; this condition may progress to rheumatoid arthritis.

MIGRATORY ARTHRITIS

Migratory arthritis is characterized by rapid onset of swelling in one or two joints, with resolution over a few days. As the symptoms resolve, similar symptoms emerge in another joint, usually in an asymmetric location.[20,28] This symptom pattern can occur in gonococcal arthritis, rheumatic fever, sarcoidosis, systemic lupus erythematosus, Lyme disease, bacterial endocarditis, and Whipple's disease.[32]

Patient Demographics

GENDER

Before menopause, women are nine times more likely to develop systemic lupus erythematosus and three to four times more likely to develop rheumatoid arthritis.[20] After men and women reach 50 years of age, the gender difference for systemic lupus erythematosus and rheumatoid arthritis becomes less significant.[1]

Compared with men, women are nine times more likely to develop fibromyalgia. An estimated 60 percent of women with symptomatic human parvovirus B19 infection manifest arthropathy, whereas men with this infection appear to develop arthropathy much less often.[33,34] The gender ratio is more balanced for spondyloarthropathies and vasculitic conditions such as polyarteritis nodosa.

Gout usually presents about 20 years after puberty in men and about 20 years after menopause in women. This disease is rare in premenopausal woman, unless renal insufficiency is present.[10]

AGE

Certain diagnoses are more common in specific age groups. Rheumatic fever, systemic lupus erythematosus, rheumatoid arthritis, reactive arthritis, and spondyloarthropathies occur more often in younger persons. Osteoarthritis, polymyalgia rheumatica, and giant cell arteritis are more common in older persons.[13]

TABLE 5

Findings of Laboratory and Imaging Tests and Associated Conditions That Result in Polyarticular Joint Pain

Laboratory or imaging test	*Condition*
Complete blood count	
Anemia	Many inflammatory arthritides, especially SLE, RA, IBD, and human parvovirus B19 infection
Thrombocytopenia	SLE, human parvovirus B19 infection
Thrombocytosis	Acute-phase reaction, vasculitis, infection
Leukopenia	SLE, RA, Felty's syndrome, Sjögren's syndrome, human parvovirus B19 infection
Leukocytosis	RA, vasculitis, reactive arthritis, infection
Eosinophilia	SLE, RA, IBD, sarcoidosis, dermatomyositis, scleroderma, Churg-Strauss syndrome, PAN, eosinophilic fasciitis, cholesterol emboli
Chest radiograph	
Infiltrates or nodules	RA, sarcoidosis, Wegener's granulomatosis, Churg-Strauss syndrome
Serositis	SLE, RA
Upper lobe fibrosis	Ankylosing spondylitis
Diffuse fibrosis	RA, scleroderma, polymyositis
Rheumatoid factor	Healthy persons; RA, SLE, Sjögren's syndrome, sarcoidosis, reactive arthritis, PMR, polymyositis, psoriatic arthritis, endocarditis, chronic infections, cancer, chronic liver disease, many nonrheumatic causes
Joint aspiration	
Culture	Infection
Crystals	Gout, pseudogout
White blood cell count	Inflammation: > 2,000 per mm^3 (2×10^9 per L)
	Probable infection: > 50,000 per mm^3 (50×10^9 per L)
Inflammatory markers: elevated erythrocyte sedimentation rate or C-reactive protein (CRP)	Infection, most inflammatory arthritides, advanced age, PMR, giant cell arteritis, cancer, anemia, pregnancy; menses
Antinuclear antibody	Healthy persons; SLE, RA, scleroderma, Sjögren's syndrome, vasculitis, polymyositis, medications, many nonrheumatic causes
Hepatic transaminase: elevated aspartate transaminase or alanine transaminase	SLE, PAN, sarcoidosis, hemochromatosis, Sjögren's syndrome, infectious hepatitis, polymyositis
Urinalysis	
Hematuria	SLE, Wegener's granulomatosis, PAN
Proteinuria	SLE; Wegener's granulomatosis, amyloidosis
Elevated alkaline phosphatase	Bone metastases, Paget's disease, osteomalacia, PMR, ankylosing spondylitis, hyperparathyroidism
Electrocardiogram: atrioventricular block	Lyme disease, neonatal lupus, ankylosing spondylitis
Double-stranded DNA	SLE, especially lupus nephritis
Anti–SS-A (anti-Ro) and anti–SS-B (anti-La) antibodies	Sjögren's syndrome, SLE; healthy persons
HLA-B27	Healthy persons; spondyloarthropathies, reactive arthritis
Elevated uric acid	Gout, psoriatic arthritis, Paget's disease; healthy persons
False-positive VDRL	SLE, anticardiolipin antibody syndrome
Cytoplasmic antineutrophil cytoplasmic autoantibody (c-ANCA)	Wegener's granulomatosis
Elevated creatinine	SLE, Wegener's granulomatosis, vasculitis
Elevated creatine kinase (CPK)	Polymyositis, dermatomyositis, hypothyroidism
Elevated calcium	Hyperparathyroidism, cancer, sarcoidosis

SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; IBD = inflammatory bowel disease; PAN = polyarteritis nodosa; PMR = polymyalgia rheumatica.

RACE

Polymyalgia rheumatica and Wegener's granulomatosis are more likely to affect whites.[13] In contrast, sarcoidosis and systemic lupus erythematosus are more common in blacks.

FAMILY HISTORY

Familial aggregation occurs in some arthritic diseases, such as spondyloarthropathies, rheumatoid arthritis, and Heberden's nodes of osteoarthritis.[1] There is a particularly strong association between ankylosing spondylitis and the HLA-B27 allele.

Laboratory Investigations

As previously noted, many rheumatologic laboratory tests must be interpreted in the context of the individual patient. For example, antinuclear antibody (ANA) tests are positive in 5 to 10 percent of the general population, a rate

TABLE 6
Categorization of Synovial Fluid

Categorization	*White blood cell count*	*Polymorphonuclear neutrophilic leukocytes*	*Examples*
Normal	0 to 200 per mm^3 (0 to 0.2 × 10^9 per L)	<25% (0.25)	—
Noninflammatory	<2,000 per mm^3 (2 × 10^9 per L)	<25% (0.25)	Osteoarthritis, internal derangement, myxedema
Inflammatory	2,000 to 50,000 per mm^3 (2 to 50 × 10^9 per L)	>75% (0.75)	Rheumatoid arthritis, psoriatic arthritis, gout, pseudogout, *Neisseria gonorrhoeae* infection
Septic	>50,000 per mm^3 (50 × 10^9 per L); usually >100,000 per mm^3 (100 × 10^9 per L)	Usually >90% (0.90)	Septic arthritis (primary concern); occasionally, gout, pseudogout, reactive arthritis, Lyme disease

Information from reference 37.

that increases with age. Thus, given a one in 20 frequency for ANAs and a one in 2,000 frequency for systemic lupus erythematosus, only one in 100 persons with a positive ANA test will have the disease. Consequently, positive ANA test results must be interpreted with caution *(Table 3)*.[4-7] Given the high sensitivity of the currently used substrate for testing, a negative ANA test essentially rules out systemic lupus erythematosus.[1,5]

Spondyloarthropathies affect fewer than 1 percent of the general population. Indeed, patients who are HLA-B27 positive and do not have a family history of ankylosing spondylitis have only a 2 percent risk of developing this disorder.[35] Spondyloarthropathies can be overdiagnosed by relying only on a positive HLA-B27 test, because this test is positive in 8 percent of white persons.[35]

Rheumatoid factor testing lacks both sensitivity and specificity: the test is positive in 5 to 10 percent of the general population and negative in approximately 20 percent of persons with rheumatoid arthritis.[36] Therefore, both positive and negative rheumatoid factor test results must be interpreted cautiously. Indeed, rheumatoid factor testing is not useful when a patient lacks other diagnostic criteria for rheumatoid arthritis, especially synovitis.[36] The American Rheumatology Association's revised diagnostic criteria for rheumatoid arthritis use findings from the history, physical examination, and laboratory tests.[4] These criteria, which have been shown to be 91 to 94 percent sensitive and 89 percent specific, are useful for establishing a diagnosis of rheumatoid arthritis.[4,12,15]

A complete blood count, urinalysis, and a metabolic panel may provide more useful diagnostic clues than classic rheumatologic laboratory tests *(Table 5)*. For instance, hematuria, proteinuria, a low white blood cell (WBC) count, and thrombocytopenia may indicate the presence of systemic lupus erythematosus. Anemia with a low mean corpuscular volume may be a sign of underlying inflammatory bowel disease that is causing chronic gastrointestinal blood loss. Human parvovirus B19 infection can induce a decrease in the reticulocyte count, followed by anemia and, occasionally, leukopenia and thrombocytopenia.[30,34]

Synovial fluid analysis is performed primarily to diagnose infection or a crystal-induced arthritis. A synovial fluid WBC count of at least 2,000 per mm^3 (2 × 10^9 per L) suggests inflammation, whereas a count higher than 50,000 per mm^3 (50 × 10^9 per L) typically indicates synovial infection *(Table 6)*.[37] Fluid with a highly elevated WBC count or a predominance of neutrophils should be cultured to exclude infection.

Diagnostic Imaging

A number of radiographic findings are characteristic of specific rheumatic disorders. For instance, sacroiliitis is indicative of ankylosing spondylitis, erosions with periarticular osteopenia are typical of rheumatoid arthritis, and "pencil-in-cup" deformities are a sign of psoriatic arthritis. However, these radiographic findings take months to develop; early in the process, radiographs may be normal or show only nonspecific changes.

In early rheumatoid arthritis, magnetic resonance imaging demonstrates cartilage damage that is not evident on plain-film radiographs.[38] This damage highlights the importance of diagnosing rheumatoid arthritis early on the basis of the history and physical examination so that disease-modifying treatment can be initiated.

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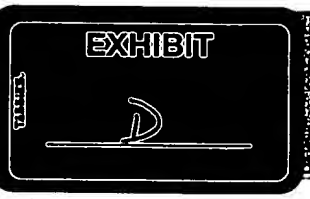

A SYNDROME OF MEGALOBLASTIC ANEMIA, IMMUNODEFICIENCY, AND EXCESSIVE NUCLEOTIDE DEGRADATION

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INTRODUCTION

Several defects of purine and pyrimidine metabolism have been associated with behavioral abnormalities The most common and best studied is Lesch-Nyhan syndrome[1], with its characteristic aggressive, self-mutilating behavior. Deficiency of adenylosuccinate lyase[2] has been reported to be associated with infantile autistic behavior. Autistic behavior as well as seizures have been associated with thymine-uraciluria[3] We report here a syndrome which involves behavioral abnormalities, seizures, and macrocytic anemia and which is associated with increased degradation of purine and pyrimidine nucleotides

CASE HISTORY

The patient, a three-year-old white female, was first seen because of recurrent infections, developmental delay, and seizures. Upon examination, she was found to have mild immunodeficiency, macroycytic anemia, ataxia, and alopecia. Physical development and speech were notably delayed IgG was borderline to low, and MCV was variable from 90 to 100 (normal for age and sex <88) A severe, recurrent sinus infection required surgical drainage. No abnormalities of amino acid or organic acid metabolism were identified by a routine metabolic screen and amino acid analysis. No unusual compounds were detected in plasma or urine by HPLC. All parameters of folic acid and B12 metabolism were found to be within normal limits The most striking feature of her phenotype was her bizarre, and often aggressive behavior She was hyperactive, with a short attention span, inappropriate verbalizations, and poor interaction with other children. Aggressive behavior took the form of pinching or scratching others, or biting toys. She would sometimes bang her head or poke at her eye with her finger. She had 2-3 seizures of 1-2 min duration per day. Initially, she was treated with IgG, folinic acid, depakote, and tegritol Her sinus infection resolved and seizure activity decreased but there was no change in her MCV, behavior, or speech development At this time, an investiga-

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tion of her nucleotide metabolism in cultured fibroblasts was begun. Based on the findings of these studies, a trial with oral nucleotides was begun Upon initiation of this treatment. an almost immediate improvement in speech, behavior, and cognitive function was seen. Speech became more understandable and appropriate and she seemed to pay more attention to her surroundings. and focus better on tasks Her interactions and play with other children became appropriate, and her mother described her behavior as that of a normal child. MCV remained elevated. Seizure activity decreased markedly, such that she was taken off depakote (625 mg/day). and the dose of tegritol was gradually reduced from 500 to 50 mg/day, with the intention of eliminating it as well. However. an interuption in the supply of nucleotides caused a one week interuption in oral nucleotide therapy. During this time. seizure activity increased to >10 seizures per day Her attention span became limited, and her frustration tolerance low. Verbalization and interaction with others deteriorated. and behavior became more aggressive At that time she was returned to pretreatment doses of depakote and tegritol. Upon resumption of nucleotide therapy, these symptoms resided. and her condition prior to the interuption of therapy gradually returned

MATERIALS AND METHODS

Incorporation studies were done as described earlier[4] for adenine, guanine, hypoxanthine, formate, uridine, and thymidine For glycine and orotic acid incorporation studies, isotope (10 uCi/ml) was added to Minimal Essential Medium and cells were grown in 75 mm plates for 72 hr, harvested by trypsinization, and analyzed by HPLC as above. Incorporation experiments were done in duplicate. For the assay of individual enzymes, cultured fibroblasts were harvested in the log phase of growth and lysed at a concentration of approximately 1 mg/ml in a 0 10 sodium phosphate buffer, pH 7.2 containing 0.05 M magnesium chloride. Serial dilutions of this lysate were incubated with a 10 uM concentration of radiolabeled substrate for 1 hr at 37°C For the assay of UMP synthetase 1 mM PRPP was added. For the assay of uridine kinase, 1 mM ATP was added The assays were deproteinized and analyzed by HPLC. Assays were done in triplicate. Reported values do not necessarily represent maximum enzyme activities due to the low substrate concentration. Nucleoside inhibition of erythroid colony formation by bone marrow cells was done as described earlier[5]

RESULTS AND DISCUSSION

The incorporation of purine and pyrimidine precursors into nucleotides is shown in Table 1 Normal incorporation of glycine into purines. as well

Table 1. Incorporation of Precursors into Nucleotides

Precursor	Patient	Controls (n)
adenine	8160	9727 (4)
hypoxanthine	3308	2849 (4)
guanine	3392	3129 (4)
formate + AICAR	3688	3658 (4)
glycine	10071	8350 (2)
uridine	3469	8511 (4)
thymidine	1223	1027 (2)
orotic acid	5694	18315 (2)

Incorporation is reported in units of pmol/100 nmol purines/2 hr

as normal excretion of uric acid by the patient indicate normal denovo purine synthesis. From the incorporation of adenine, hypoxanthine, and guanine into the various purine nucleotides it is clear that the activities of enzymes of purine nucleotide interconversion (i e. adenylosuccinate synthetase, adenylosuccinate lyase, AMP deaminase, IMP dehydrogenase GMP. synthetase, GMP reductase, and the purine nucleotide mono- and diphosphate kinases) are comparable to those of normal controls (data not shown). Similarly, the production of normal amounts of UTP, CTP, and TTP from uridine indicates that the enzymes of pyrimidine nucleotide interconversion (i e. CTP synthetase, thymidylate synthetase, and the pyrimidine mono- and diphosphate kinases) are comparable to normal controls. The only notable differences in these precursor incorporation studies was the low incorporation of orotic acid and uridine into pyrimidine nucleotides This could reflect low activities of the synthetic enzymes or increased catabolism of the nucleotide products.

To study this question further, individual enzyme activities in dialyzed fibroblast lysates were measured (Table 2) To determine if a deficiency existed in the synthesis of pyrimidine nucleotides, the activities of UMP synthetase and uridine kinase were measured, and found to be normal. The only consistent difference between the patient and normal controls was a ten- to thirty-fold increase in the catabolism of UMP. When the rate of purine nucleotide catabolism was measured for comparison a similar elevation was found. Interestingly, the increased catabolism of purine nucleotides had no noticeable effect on the incorporation of purine precursors into purine nucleotides, whereas the increased catabolism of pyrimidine nucleotides appeared to result in a net decrease in pyrimidine nucleotide synthesis, as measured in cultured fibroblasts To further study the metabolism of pyrimidine nucleotides in intact cells, the effect of pyrimidine nucleosides on erythroid colony formation was measured in the presence and absence of nucleosides (Table 3) Clearly, the patient's bone marrow cells show much less inhibition of colony formation in presence of thymidine and uridine than control bone marrow cells. This indicates that in the patient's cells, pyrimidine nucleosides are either transported or phosphorylated more slowly, or that the pyrimidine nucleotides, once formed, are degraded more rapidly. Again, it is interesting to note that this effect is present with thymidine, although intact fibroblasts show no decrease in the incorporation of thymidine into thymidine nucleotides

On the basis of these results, it was decided to intiate pyrimidine nucleotide replacement therapy. In orotic aciduria, in which there is a known defect in pyrimidine synthesis associated with macrocytic anemia, pyrimidine nucleotide replacement therapy has been quite successful. The patient was started with 150 mg/kg/day each of UMP and CMP Plasma uri

Table 2 Activities of Catabolic Enzymes in Dialyzed Cell Lysates

Enzyme (substrate)	Patient	Controls (n)
5'Nucleotidase (UMP)	7 44	0.65 (4)
5'Nucleotidase (AMP)	9.64	0.80 (4)
Nucleoside Phosphorylase (uridine)	0 41	0 44 (4)
Nucleoside Phosphorylase (inosine)	5 72	4 24 (4)
Adenosine Deaminase	3 54	5 09 (4)
UMP Synthetase	1 56	2 17 (4)
Uridine Kinase	2.86	3 51 (2)

Enzyme activities are in nmol/min/mg protein

Table 3. Inhibition of Erythroid Colony Formation by Nucleosides

Nucleoside (concentration)	Patient	Control
None	347 (100%)	169 (100%)
Uridine (10 uM)	322 (94%)	166 (98%)
Uridine (50 uM)	320 (94%)	164 (97%)
Uridine (75 uM)	335 (98%)	56 (33%)
None	508 (100%)	328 (100%)
Thymidine (10 uM)	486 (95%)	251 (76%)
Thymidine (50 uM)	298 (59%)	0 (0%)
Thymidine (75 uM)	96 (18%)	0 (0%)

Colony formation in units of average colonies per plate

dine and erythrocyte UTP were monitored during therapy Plasma uridine was undetectable before therapy and stayed in the range of 20-50 uM during therapy. Erythrocyte UTP was similarly undetectable before therapy, and was maintained in the range of 20-60 nmol/ml packed red cell during therapy A general improvement in the patient's condition was noted, but MCV remained abnormally high Because increased catabolism of purine nucleotides was also indicated. 75 mg/kg/day AMP was included, and the daily dose of pyrimidines was increased to 500 mg/kg/day These measures produced no additional improvement, and MCV remained high.

At present. the precise metabolic basis of these symptoms remains unknown The increase in nucleotidase activity could be the primary defect, or it could be a response to abnormal amounts of some as yet unidentified nucleotide. The fact that MCV remained high. even during nucleotide replacement therapy with adenine. cytidine, and uridine nucleotides might indicate a shortage of other nucleotides. perhaps deoxynucleotides. Alternatively, the amount of nucleotides used here may have been inadequate to maintain normal nucleotide levels in the presence of increased nucleotidase activity

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MITOCHONDRIAL ENCEPHALOMYOPATHIES: WHERE NEXT?

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Introduction

There are papers in the history of medicine that will never become obsolete because they combine original thinking with the best technology available at the time to open up new vistas. One such paper is the report in 1962 by Luft and coworkers of a young Swedish woman with severe hypermetabolism, mild weakness, and normal thyroid function (Luft, et al. 1962). The genial intuition of Rolf Luft, an endocrinologist, that the problem was to be sought in skeletal muscle rather than in the thyroid gland, combined with the expertise in mitochondrial bioenergetics of the late Lars Ernster and the morphological acumen of Bjorn Afzelius, led the Karolinska group to discover the first mitochondrial disease and the first example of "organellar medicine". The fact that Luft disease is one of the rarest human metabolic disorders is a historical curiosity that does not diminish the importance, indeed the beauty, of that observation.

What Luft may not have suspected at the time was that he was opening a seemingly bottomless Pandora's box of human diseases. By 1994, it was clear that mitochondrial dysfunction could affect every tissue in the body, thus amply justifying the term "mitochondrial medicine" that Luft introduced in the title of a review article (Luft 1994). At this meeting, he gave us a fascinating account of the discovery of Luft disease and an update on "mitochondrial medicine".

Of course, one of the unique characteristics of mitochondrial diseases is that mitochondria are relics of independent bacteria-like intruders (welcome intruders, as it tumed out) that took permanent residence in our cells over a billion years ago. As such, mitochondria possess their own DNA (mtDNA) and are under dual genetic control. If we focus our attention on the "business end" of mitochondrial energy metabolism, the respiratory chain, only 13 of its approximately 90 proteins are encoded by mtDNA and are synthesized within the organelle, whereas all others are encoded by nuclear DNA (nDNA). After being synthesized in the cytosol, the nDNA-encoded subunits are imported into the organelle, where they are assembled, together with their mtDNAencoded counterparts, into the respective holoenzymes in the mitochondrial inner membrane.

Although mtDNA was discovered 36 years ago (Nass and Nass 1963) and human mtDNA had been fully sequenced by 1981 (Anderson, et al. 1981), clinicians paid no attention to this genetic relic until 1988, when mutations in mtDNA were first associated with human disease; (Holt, et al. 1988, Wallace, et al. 1988). In the intervening years, however, a cadre of scientists had worked hard at clarifying the organization of the mitochondrial genome and the peculiar rules governing its replication, transcription, and translation (Schon 1997). One of the pioneers in this field was Giuseppe Attardi at the California Institute of Technology. His participation in this meeting illustrates another interesting aspect of mitochondrial pathology: the close collaboration between basic and clinical scientists. The lack of spontaneous or engineered animal models for mtDNA related diseases has made patients and patient-derived celis precious commodities where concepts derived from *in vitro* studies could be verified *in vivo* or, at least, in tissue culture, such as the cybrid system introduced by King and Attardi (King and Attardi 1989).

As the title of this chapter implies, we will not even attempt to review the enormous progress achieved in the 38 years since Luft and coworkers introduced the concept of mitochondrial disease, nor even in the 12 years since the description of pathogenic mtDNA mutations. In trying to gaze into the future, we will discuss four

subjects of special interest to our group, where recent information has made us revise old "dogmas" or has opened up new areas of investigation. In keeping with the concept that the term "mitochondrial encephalornyopathy" refers to defects of oxidative phosphorylation, and that the respiratory chain is under the control of two genomes, we will consider two subjects related to mtDNA and two related to nDNA.

I. Have we neglected the mtDNA protein-coding genes?

During the past 12 years, experience with a rapidly increasing population of patients harboring mtDNA mutations has led clinical investigators to formulate a number of generalizations that are too often interpreted as "dogmas". Following is a short list of such dogmas.

1. Point mutations in mtDNA are transmitted by maternal inheritance and negative family history is evidence against such etiology.

2. mtDNA point mutations are associated with multisystem disorders and are rarely strictly tissue-specific.

3. Ragged-red fibers (RRF), the histochemical hallmark of massive mitochondrial proliferation in muscle, are typically seen in patients with mtDNA mutations that impair overall mitochondrial protein synthesis, such as mutations in tRNA or rRNA genes, single or multiple deletions, or mtDNA depletion. Conversely, RRF are absent in muscle biopsies from patients with mutations in mtDNA protein-coding genes, such as the NARP/MILS mutations in the ATPase 6 gene, or the various mutations in genes encoding complex 1 subunits (ND genes) associated with Leber's hereditary optic neuropathy (LHON).

4. In patients with mtDNA point mutations, RRF do not show cytochrome c oxidase (COX) activity (i.e. are histochemically COX-negative), except in patients with typical MELAS syndrome, where they are COX-positive. All of these dogmas have been shattered, or at least cracked, by recent experience.

1. Point mutations in protein-coding genes often arise de novo and cause sporadic disorders. Examples abound and are illustrated in Tables 1 and 2.

Among *complex* I gene defects, only two have been found in sporadic cases thus far: a nonsense mutation in the ND4 gene (Andreu, et al. 1999c), and an intragenic inversion in the NDI gene altering three highly conserved amino acids (Musumeci et al, in preparation). Both patients suffered from exercise intolerance and one had minimal proximal limb weakness.

Mutations in cytochrome *b,* the only mtDNA-encoded subunit of *complex III,* offer the best example of this apparently anomalous situation (jable 1). Nine sporadic patients, all with exercise intolerance and two with recurrent myoglobinuria, had pathogenic mutations in the cytochrome *b* gene (Dumoulin et al. 1996; Andreu et al. 1998; Kennaway et al. 1998; Andreu et al. 1999a; Andreu, et al. 1999b). All mutations were different, though all were G-to-A transitions. However, not all pathogenic mutations in the cytochrome *b* gene are spontaneous events: a maternally inherited multisystem disorder with features of MELAS and parkinsonism was associated with a microdeletion (De Coo et al. 1999).

When it comes to defects of *complex* IV (cytochrome c oxidase, COX), the variety of clinical presentations is greater, but, again, maternal inheritance is extremely rare, having been seen in only one of nine reported patients (Table 2).

2. Point mutations in protein-coding genes are often tissue-specific.

The one tissue that is most often affected is skeletal muscle, suggesting that these are somatic mutations, that is, spontaneous events that arose in myoblasts or in myoblast precursors after germ-layer differentiation. Pure myopathy, dominated by exercise intolerance with or without myoglobinuria and sometimes associated with mild limb weakness, characterized two patients with *complex I deficiency,* all nine patients with *complex III deficiency* (jable 1), and two patients with COX *deficieney*

(jable 2).

Exercise intolerance is a common complaint, which, if the patient has no objective weakness, increased serum creatine kinase (CK) levels, or abnormal electromyography (EMG), is often dismissed as "psychogenic" or mislabeled as "chronic fatigue syndrome"or "fibromyalgia rheumatica". Many patients with mutations in mtDNA protein-coding genes fall into this group, and the lack of maternal inheritance further distracts the physician from thinking about mitochondrial dysfunction. It is important, when faced by these puzzling patients, to consider the possibility of a mitochondrial disease and to obtain, at the very least, a resting lactate value. Increased lactate was the only abnormality that led an astute clinician to biopsy a young man with life-long exercise intolerance. The muscle biopsy showed RRF and markedly decreased complex I activity, and molecular genetic analysis of muscle mtDNA revealed a- nonsense mutation in the ND4 gene(Andreu et al. 1999c).

Myoglobinuria, and especially recurrent myoglobinuria, is commonly associated with blocks in the utilization of the two major sources of energy for muscle contraction, glycogen or fatty acids (DiMauro and Haller 1999). Strangely, blocks in oxidative phosphorylation, the final common pathway for energy production, were not considered until recently in the differential diagnosis of myoglobinuria. However, two patients with *complex III deficiency* did have each one episode of myoglobinuria (Andreu et al. 1999a, Andreu et al. 1999b) whereas both patients with myopathy and COX *deficiency* had multiple episodes of myoglobinuria related to unusually intense or repeated exercise ; Karadimas et al. 1999). The difference in the frequency and intensity of myoglobinuria attacks between patients with defects in complex HI and IV suggests that COX deficiency causes a more severe "energy crisis". For the sake of completion, we should mention one other respiratory chain defect often associated with myoglobinuria, coenzyme Q10 (CoQ10) deficiency (Ogasahara et al. 1989, Servidei et al. 1996, Sobreira et al. 1997). However, CoQ10 is not encoded by mtDNA and primary defects of CoQ10 are presumably due to mutations in

nuclear genes encoding one or more biosynthetic steps.

In two patients with sideroblastic anemia and COX deficiency, distinct but closely located mutations in the COX 1 gene were found in bone marrow, whole blood, isolated platelets, and granulocytes, but not in T or B lymphocytes, buccal mucosa, or skin fibroblasts (Gattermann et al. 1997). This pattern suggested that the mutations occurred *de novo* in bone marrow stem cells with myeloid determination. The fact that tissues other than muscle can be selectively affected suggests that we should keep an open mind about the possibility that somatic mutations of mtDNA protein-coding genes- may be involved in other tissue-specific disorders, such as cardiopathies or encephalopathies.

3. Ragged-red fibers are commonly associated with mutations in protein-coding genes. This is especially evident in patients with complex I and complex In deficiencies. Both patients with *complex I deficiency* (Andreu et al. 1999c), and all but one patient with *complex III deficiency* (Table 2) had RRF, which stained intensely for COX (COX positive RRF). The only exception was a 38-year-old wornan. with exercise intolerance, proximal weakness, and a missense mutation in the cytochorne *b* gene (Andreu, et al. 1998). In patients with *complex IV deficiency,* RRF are generally less abundant and can even be absent. Not surprisingly, those RRF that there are, are COX-negative, and there is an abundance of COX-negative non-ragged-red fibers. These findings cast some doubts on the hypothesis that impairment of mitochondrial protein synthesis is the trigger for mitochondrial proliferation.

2. Why is MELAS different from KSS?

This "tongue-in-cheek" question actually reflects our woeful ignorance about the pathogenesis of mtDNA-related diseases, and especially those due to mtDNA rearrangements and to mutations in tRNA genes. There is ample evidence from studies of patients' tissues and of cybrid cell lines that both types of mutations impair mitochondrial protein synthesis and affect one and the same pathway, the

respiratory chain. Why, then, are there so many different clinical presentations, some of which are stereotypical enough to be easily recognizable at the bedside and amenable to acronymic labeling, such as MELAS, MERRF, or KSS? Some answers are provided by the peculiar rules of mitochondrial genetics. Thus, varying degrees of heteroplasmy in different tissues coupled with different thresholds of vulnerability to oxidative iMpairment can explain much of the inter-organ variability in these disorders.

However, questions become harder when it comes to the brain. Why are certain brain functions typically affected in MELAS while others are predominantly affected in MERRF and still others in KSS? For example, the stroke-like lesions of MELAS are only rarely seen in MERRF or KSS; conversely, myoclonus is fairly typical of MERRF; and seizures are virtually obligatory in both MELAS and MERRF but are very rare in KSS. Given the cellular heterogeneity and organizational complexity of the brain, we have posited that uneven spatial degrees of heteroplasmy for different mutations might explain some of the differential neurological signs. With this working hypothesis, we have started to draw "morbidity maps" of the brain in the different mitochondrial encephalomyopathies based on comparative immunohistochemistry, using antibodies against mtDNA-encoded respiratory chain proteins (e.g. COX H or NDI) and nDNAencoded proteins (e.g. COX IV or the non-heme iron-sulfur protein of complex 111).

This approach is yielding some interesting results. When we studied the dentate gyrus of the hippocampus, a highly epileptogenic area (Dichter and Buchhalter 1997), from. two patients, one with MELAS the other with KSS, we found that immunoreactivity for the FeS-protein was similar in both whereas immunoreactivity for COX II was markedly reduced in the patient with MELAS but normal in the patient with KSS (Figure 1). Although indirect, these data suggest that the A3243G MELAS mutation was abundant enough in the dentate gyrus to curtail mitochondrial protein synthesis, while the mtDNA deletion in the KSS patient must have been below the threshold needed to affect COX H biosynthesis. An even more indirect,

but not unreasonable, conclusion is that the relative sparing of the hippocampus in KSS may contribute to explain the rare occurrence of seizures in this condition.

We next asked ourselves if a sirnilar spatially selective nútochondrial dysfunction could explain cerebellar ataxia, another common symptom of mitochondrial encephalomyopathies, and especially of MERRF and KSS.

In normal brains, immunohistochemistry of the cerebellar system with anti-COX II and anti-FeS antibodies showed finely punctate reaction in the cortex, the dentate nucleus, and the olivary nucleus. In the cerebellar cortex, the immunostain was evenly distributed throughout the molecular layer, showed a dense granular pattern in the glomeruli of the internal granular layer, and decorated clearly both perikaria and apical dendrites of Purkinje cells. The shapes of the dentate and olivary nuclei were neatly outlined by the immunoreaction of the neuropil, and large multipolar neurons reacted strongly with both antibodies (Figures 2, 3, and 4 .

In both MERRF and KSS, the immunoreactivity of the cerebellar cortex was similar to that of control brains with both antibodies (Figure 2). In KSS, however, immunostain with COX II antibodies was markedly decreased in the dentate nucleus, in contrast to the normal reaction with FeS antibodies ffigure 3). In MERRF, this selective immunostaining defect for the mtDNA-encoded protein included not only the dentate but also the olivary nucleus (Figures 3 and 4). Thus, we have strong immunochernical evidence of mitochondrial dysfunction in cerebellar structures that may play an important role in the pathogenesis of ataxia. The dentate nucleus is an important relay station in the cerebello-thalamo-cortical pathway, which receives axons from the Purkinje cells of the cerebellar hemispheres. The inferior olivary nucleus appears to be the pacemaker station of the cerebellar system, where climbing fibers originate on their way to innervate Purkinje cells. Our evidence that the A8344G MERRF mutation accumulates in both dentate and olivary nuclei while deleted mtDNAs are especially abundant in the dentate nucleus suggests that there may be a major disconnection of the cortico-nuclear cerebellar

system at least at the level of the dentate nucleus. This disconnection may play a major role in the pathogenesis of ataxia in patients with KSS and MERRF (Tanji et al. 1999).

Although some interesting immunopathological brain "rnaps" are emerging, this still begs the question of which factors are responsible for the putative selective spatial abundance of distinct mutations in different areas of the brain.

3. Mitochondrial diseases: back to mendelian genetics

For the past decade and until recently, mtDNA-related disorders seemed to be "the only game in town": novel mtDNA mutations were described seemingly every week, and the clinical expression of mtDNA mutations was fascinating in its variety. As discussed above, much remains to be done before we fully understand the pathogenetic mechanisms of mtDNA mutations. However, mtDNA is small and we may be scraping the bottom of the barrel searching for new mutations. The time has come to direct our attention to the more difficult task of identifying nuclear DNA mutations responsible for respiratory chain defects. This is particularly important because two of the four most common causes of Leigh syndrome, a devastating neurodegenerative disease of infancy or childhood, aredue to specific respiratory chain defects: complex I deficiency (Rahman et al. 1996; Kirby et al. 1999), and COX deficiency (Willems, et al 1977; DiMauro et al. 1987; Van Coster et al. 1991). Both conditions are inherited as autosomal recessive traits. The other two common causes of Leigh syndrome are pyruvate dehydrogenase complex (PDHC) deficiency and the T8993G mutation in the mtDNA ATPase 6 gene. PDHC deficiency is usually inherited as an X-linked dominant trait while the T8993G mutation is the most common cause of maternally inherited Leigh syndrome (MILS).

This shift in interests towards the nuclear genome is yielding exciting results, and it has generated fancifully titled editorials, such as "Nuclear power and mitochondrial

each candidate gene is a tedious and uncertain task, and because LS families are too small for traditional linkage analysis, Shoubridge's group resorted to functional complementation of the enzyme defect in cells from LS patients, using microcell-mediated chromosome transfer. They showed that transfer of chromosome 9 into COX-defective cells restored COX activity, and, using deletion mapping, localized the genetic defect to a 4.5 cM region of chromosome 9q34, containing the candidate gene *SURF-1*. Sequence of *SURF-1* in patients revealed five different pathogenic mutations, all of which predicted a truncated protein (Zhu et al. 1998). Soon thereafter, and using a similar approach, the group of Zeviani identified five more distinct mutations in the same gene (Tiranti et al. 1998).

The product of *SURF-1* is indeed a mitochondrial protein, which appears to act at the third stage in the four-step process of COX assembly (Zeviani et al. 2000). Northern and Western blot analyses in tissues from patients with *SURF-1* mutations failed to show any COX transcript or protein, suggesting that these loss-of-function mutations are associated with mRNA instability, rapid protein degradation, or both (Zeviani et al. 2000).

Although the discovery of *SURF1* mutations in COX-deficient LS was a major breakthrough, the story clearly does not end there, because these mutations were not found in all patients. Besides, we had known all along that COX-deficient LS was clinically and biochemically heterogeneous: for example, we had seen a few patients with severe congenital cardiomyopathy in whom COX activity was only partially decreased in liver and fibroblasts (DiMauro, et al. 1987). We decided, therefore, to study COXassembly genes other than *SURF1* in these unusual patients, and we found pathogenic mutations in the SC02 gene in three infants with fatal infantile cardiomyopathy and encephalopathy, but without the typical neuropathological features of LS (Papadopoulou et al. 1999). The SC02 gene encodes a copper-binding protein that must play a crucial role in the assembly of COX, which contains two copper atoms. This essential function coupled with Northern blot evidence that the SC02 protein is expressed predominantly in heart and muscle explains both the

clinical phenotype and the extremely low levels of COX activity found in heart and muscle.

We have screened our series of 41 patients with undiagnosed encephalomyopathies and COX deficiencies for *SURF-1* and SC02 mutations (Sue et al. 2000). We found six patients with *SURF-1* mutations and three with SC02 mutations, and a comparison of the two groups revealed some interesting distinguishing features. First, at the clinical level, all patients with SURF-1 mutations had typical clinical and pathological features of LS, whereas all three patients with SC02 mutations died in early infancy of fulminant cardiopathy. Second, histochemistry of muscle biopsies showed much more severe COX deficiency in patients with SC02 mutations, a finding confirmed by biochemical assays. Third, immunohistochemistry indicated a similar decrease of nDNA-encoded and mtDNA-encoded COX subunits in *SURF-1* patients, but a more severe decrease of mtDNA-encoded proteins in SC02 patients.

It is noteworthy that only 14% of our patients with COX-deficient LS had *SURF1* mutations, in sharp contrast to 75% in Tiranti's series (Tiranti et al. 1999). The two main reasons for this discrepancy may be that Tiranti's patients belonged to a single complementation group (Munaro et al. 1997) and to a relative uniform ethnic group.

Irrespec tive of their exact frequency, knowledge of two genetic defects associatedwith COX-deficient LS syndrome will now make prenatal diagnosis possible for parents who have often lost one or more children to the disease. On the other hand, the molecular defect remains unknown in a substantial number of COX-deficient LS patients. Screening additional COX-assembly genes for pathogenic mutations is the obvious next step and one that will undoubtedly bear fruit.

4. Defects of intergenomic signaling: the lesson of MNGIE

A special group of mendelian mitochondrial diseases reflects the gradual loss of autonomy of the mitochondrial genome, which now depends heavily on factors encoded by nuclear genes for some of its essential functions, including transcription,

translation, and replication. Disorders of intergenomic signaling are due to mutations in nuclear genes that, directly or indirectly, control mtDNA number, function, or integrity.

The first example of such faulty "dialogue" between the two genomes was offered by patients with autosomal dominant progressive external ophthalmoplegia (PEO) and multiple mtDNA deletions in muscle (instead of the single type of mtDNA rearrangement that characterizes each patient with KSS or sporadic. PEO) (Zeviani et al. 1989). Although linkage analysis has provided both chromosomal localization and evidence of genetic heterogeneity in some families with autosomal dominant PEO (Suomalainen et al. 1995; Kaukonen et al. 1996), no genes responsible for this syndrome have yet been identified.

The second major disorder of intergenomic communication, resulting in tissue specific paucity of mtDNA copies ("mtDNA depletion"), was described in infants with severe congenital myopathy or hepatopathy (Moraes et al. 1991). There are milder myopathic forms of mtDNA depletion (Tritschler et al. 1992) and the clinical spectrum may involve both central and peripheral nervous systems (Vu et al. 1998). The genetic defect (or defects), which presumably impair mtDNA replication, remain elusive. Decreased levels of mitochondrial transcription factor A (mtTFA) in tissues from patients (Larsson et al. 1994; Poulton et al. 1994) probably are a consequence rather than the cause of mtDNA depletion. A partial defect of polymerase -y was reported in a singlepatient with Alpers syndrome and mtDNA depletion (Naviaux et al. 1999), but polymerase y deficiency has not been found in patients with typical mtDNA depletion.

The first defect of intergenomic signaling whose molecular defect has recently been identified is an autosomal recessive form of PEO known by the cacophonic acronym MNGIE, for mitochondrial neurogastrointestinal encephalomyopathy (Hirano et al. 1994). In contrast to the autosomal dominant forms of PEO, which are largely confined to muscle, autosomal recessive PEO syndromes with multiple mtDNA

deletions tend to be multisystemic (Carrozzo, et al. 1998). This is well illustrated by MNGIE, a syndrome dominated by gastrointestinal problems (chronic diarrhea, intestinal pseudoobstruction) leading to cachexia and early death. Additional symptoms and signs includeptosis and ophthalmoplegia, peripheral neuropathy, and leukoencephalopathy. Muscle biopsy shows COX-negative RRF, biochemical evidence of COX deficiency, and molecular evidence of mtDNA multiple deletions, sometimes associated with mtDNA depletion (Bardosi et al. 1987; Hirano et al. 1994; Papadimitriou et al. 1998). First, linkage analysis of four ethnically distinct families with typical MNGIE localized the gene to chromosome 22q13.32-qter (Hirano et al. 1998). Sequencing of candidate genes in this region led to the discovery of homozygous or compound heterozygous mutations in the gene specifying thymidine phosphorylase (TP) in 12 MNGIE patients (Nishino et al. 1999). TP is widely expressed in human tissues, including some that are selectively involved in MNGIE, such as the gastrointestinal system, brain, and peripheral nerves. Paradoxically, however, TP is not expressed in skeletal muscle, which is also affected in MNGIE clinically and which harbors multiple mtDNA deletions. This suggests that the mtDNA abnormalities in MNGIE may be a secondary phenomenon, possibly related to damage by abnormal extracellular thymidine pools. One practical advantage of knowing the gene defect is the availability of a simple biochemical diagnostic assay, based on TP activity in leukocytes, which is virtually undetectable in patients (Nishino et al. 1999). Another practical implication is the possibility that correcting the imbalande of the nueleotide pools might have therapeutic value in this otherwise intractable and devastating disorder.

5. Where next?

The four subjects discussed above illustrate, we hope, both the exciting progress of research in the area of mitochondrial diseases and the challenging problems that still face us. So, where do we go next? Here are some of the many avenues wide open to the curious clinical investigator:

I. Mitochondrial genome: are we scraping the bottom of the barrel? Most emphatically, no! We have documented how mutations in protein-coding genes have been relatively neglected and we predict that more attention to these genes will yield a rich harvest.

The pathogenic mechanisms of mtDNA mutations remain largely elusive, especially the apparent "symptorn specificity" of some mutations (Schon et al. 1997).

We do not understand why RRF are much more common in patients with mtDNA than in patients with nDNA mutations. For that matter, we still do not know what triggers mitochondrial proliferation and the formation of RRF

2. Nuclear genome: the search for nuclear gene mutations causing defects in respiratory chain complexes has just started with a bang and promises to be very exciting. The experience with COX deficiency warns us about the possibility of "murder by proxy", that is, gene defects only indirectly related to the respiratory complex in question. Conversely, it is surprising that mutations in nuclear genes encoding COX subunits have not yet been found, and it is a safe bet that sooner or later some such genetie error will be associated with one or more COX-deficiency syndrome.

Nuclear genes not only codify most respiratory chain subunits and assembly factors, but also numerous factors needed for mtDNA replication and integrity. The recent discovery of the molecular basis of MNGIE bodes well for similar breakthroughs in other defects of intergenomic signaling.

Nuclear genes also encode the whole complex machinery needed for mitochondrial protein importation. Although a handful of defects in 1eader peptides", needed for the recognition of mitochondrial proteins by the organelle, have been reported (Fenton 1995), defects of the general importation system are still largely "terra incognita". However, defects of the chaperonin heat shock protein 60 (hsp60) have been described in two children with mitochondrial encephalomyopathy (Agsteribbe

et al. 1993, Briones et al. 1997), and a mutation in DDP, a protein similar to the yeast TIM (translocase of the inner membrane) family of translocases, has been identified in patients with the deafness and dystonia syndrome (Koehler et al. 1999). The stage is set for still another subgroup of mitochondrial diseascs to be defined at the clinical and molecular level.

Transport of metabolites across the inner mitochondrial membrane requires a whole battery of translocases encoded by the nuclear genome. These have been largely neglected by clinical researchers, with the exception of the carnitine-acylcamitine carrier (CAC). CAC is part of the "carnitine cycle", feeding long-chain fatty-acyl-CoAs into the mitochondrial P-oxidation pathway, and several patients with biochemically identified CAC deficiency had been reported. Only recently, however, has the gene for CAC been cloned (Huizing et al. 1997) and the first molecular defect identified in a patient with CAC deficiency (Huizing et al. 1998). In addition, reduced amounts of the voltage dependent anion channel (VDAC) of the outer mitochondrial membrane were documented by Western blot in a patient with psychomotor retardation, although the molecular defect was not elucidated (Huizing et al. 1996). Putative genetic defects of the inner membrane adenine nucleotide translocators (ANTs) would be of more direct relevance to mitochondrial disorders due to respiratory chain dysfunction. This coneept has been bome out by a knockout mouse deficient in the heart/muscle isoform of ANT (ANTI), an excellent model for human mitochondrial myopathy and cardiopathy (Graham et al. 1997).

3. Defects of the respiratory chain physical milieu. There is ample evidence that the functioning of respiratory chain complexes depends on the integrity of the phospholipid milieu in which they are embedded, especially on a normal amount and structure of cardiolipin (Schlame et al. 1999). Thus, it is at least theoretically possible that patients with multiple respiratory chain defects may have genetic or acquired abnormalities of the inner membrane phospholipids rather than mtDNA mutations. One example of this scenario may be Barth syndrome, an X-1inked recessive disorder characterized by cardiopathy, myopathy, leukopenia, and

multiple respiratory chain defects in muscle (Barth et al. 1999). Interestingly, the gene responsible for Barth syndrome,TAZ, encodes a protein called tafazzin, which has sequence homology to a superfamily of acyltransferases active in phospholipid biosynthesis (Barth et al. 1999). Although the alteration in the phospholipid composition of the inner mitochondrial membrane remains to be documented in Barth syndrome, this concept has exciting implications for this and other mitochondrial eneephalomyopathies.

4. Therapy. To the frustration of fan-ùlies and doctors, therapy of respiratory chain disorders remains woefully inadequate and usually limited to the administration of various vitamins and cofactors. Gene therapy is still a distant possibility for nuclear defects and is daunting for mtDNA gene defects. However, because of heteroplasmy and the threshold effect, if we could cause even a small shift in the relative percentages of mutant and wild-type mtDNAs, we might affect the clinical phenotype dramatically. Various strategies are being cosidered, including the use of peptide nucleic acids (PNAs) to inhibit the replication of complementary mutant mtDNAs (Taylor et al. 1997), or pharmacologic approaches directed to the same end (Manfredí et al. 1999). The observation that myoblasts, the progenitor muscle cells, often contain lesser amounts ofpathogenic mtDNA mutations than mature muscle fibers (Clark et al. 1997; Shoubridge et al. 1997) has suggested the use of exercise as a mean of inducing limited muscle necrosis, which would be followed by regeneration of muscle fibers harboring lower mutational loads (Taivassalo et al. 1999). This approach could be particularly useful in patients with protein-coding mtDNA gene mutations limited to the musculature (see above - section 1). Thus, there are some promising experimental approaches, but therapy in general remains a major challenge for future researchers.

5. Genetic counseling. This is another frustrating aspect of the mitochondrial diseases. While recent discoveries of mutations in nuclear genes responsible for Leigh syndrome or mitochondrial encephalocardiomyopathy are offering couples the option of prenatal diagnosis, this remains problematic for disorders due to mtDNA

mutations. The lack of a clear correlation between mutational loads in amniocytes or chorionic villi and other fetal tissues for the most common pathogenic tRNA mutations, such as those associated with MELAS and MERRF, makes prenatal diagnosis in these conditions impossible. Theoretically, the nucleus could be removed from the ovum of an asymptomatic or oligosymptomatic wornan harboring the MELAS mutation and transplanted into a normal enucleated oocyte. Fertilization and implantation of the manipulated ovum would result in a child with the mother's nuclear genome and the donor's mitochondrial genome (Rubenstein et al. 1995). Obviously, such germ-line therapy raises a number of experimental and ethical issues. In contrast to tRNA gene mutations, the NARP/MILS mutation (T8993G) in the ATPase 6 gene of mtDNA appears to be uniformly distributed in different tissues (White et al. 1999b), thus making prenatal diagnosis feasible (White et al. 1999a).

Clearly, mitochondrial diseases, introduced forty years ago by Luft and coworkers, remain a fertile area of investigation for clinical investigators and will keep them busy well into the new century, if not the new millennium.

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Patient	1	2	3	4	5	6	7	8	9
Sex/age	M/43	F/52	F/38	M/32	M/51	M/28	F/23	F/38	M/27
Age at onset	30 yr	childhood	childhood	childhood	childhood	25	15	25	12
Exercise int.	+	+	+	+	+	+	+	+	+
Weakness	+	+	-	-	+	+	+	+	+
Myoglobinuria	+	-	-	-	-	-	-	-	+
Family history	-	-	-	-	-	-	-	-	-
Lactic acidosis		+	+	+	+	+	+	+	+
COX+ RRF	+	+	+	+	+	+	+	-	+
cyt b mutation	24del	G14846A	G15168A	G15084A	G15723A	G15615A	G15242A	G15762A	G15059A
a.a. change	8aa del	G1y34Ser	Trpl4lstop	Trpll3stop	Trp326stop	Gly290Asp	Glyl66stop	Gly339Glu	Glyl90stop
% heteroplasmy	50	85	70	87	87	85	63	85	63

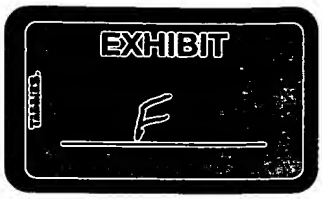
Table I. Cliffical features, muscle pathology, and molecular findings in musele mtDNA in 9 patients with exercise intolerance. Only Gomori trichrome stain was used. Patients 1-5 are from Andreu et al, 1999b; patient 6 from Dumoulin et al, 1996; patient 7 from Kennaway et al, 1998; patient 8 from Andreu et al, 1998; and patient 9 from Andreu et al, 1999.

Subunit e	Mutation	Family history	Clinical Manifestations	Reference
COX I	T6742C	negative	acquired sideroblastic anemia	Gattennan et al, 1997
COX I	T6721C	negative	acquired sideroblastic anemia	Gatterman et al, 1997
COX I	G6930A		negative deafness; ataxia; blindness	Bruno et al, 1999
COX I	bpl delta	negative	motor neuron disease	Comi et al, 1999
COX I	G5920A	negative	exercise intolerance, myoglobinuria	Karadimas et al, 1999
COX II	T7587C	maternal	myopathy, ataxia, dementia, optic atrophy	Clark et al, 1999
COX II	T7671A	negative	proximal limb weakness	Rahman et al, 1999
COX III	T9957C	negative	MELAS	Manfredi et al, 1995
COX III	5 bp delta	negative	exercise intolerance, myoglobinuria	Keightley et al, 1996
COX III	G9952A	negative	exercise intolerance, encephalomyopathy	Hanna et al, 1998

Table 2. Clinical features associated with mutations in mtDNA-encoded COX subunits.

Complex	Gene	Mutations	Clinical Picture	Reference
I	*NDUFS4*	5-bp duplication	LS-like	van den Heuvel et al, 1998
I	*NDUFS8*	P79L, RI02H	LS (cardiomyopathy)	Loeffen et al, 1998
I	*NDUFVI*	A341V	myoclonic epilepsy	Schuelke et al, 1999
I	*NDUFVI*	R59X, T423M	myoclonic epilepsy	Smeitink & van den Heuvel, 1999
I	*NDUFS7*	V122M	LS	Smeitink & van den Heuvel, 1999
II	flavoprotein	C1684T	LS	Bourgeron et al, 1995
IV	*SURF-I*	multiple	LS	Zhu et al, 1998; Tiranti et al, 1998
IV	SC02	multiple	cardioencephalopathy	Papadopoulou et al, 1999

Table 3. Molecular defects in nuclear genes causing defects in respiratory chain complexes. LS, Leigh syndrome.



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Review

The development of mitochondrial medicine

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ABSTRACT **Primary defects in mitochondrial function are implicated in over 100 diseases, and the list continues to grow. Yet the first mitochondrial defect—a myopathy—was demonstrated only 35 years ago. The field's dramatic expansion reflects growth of knowledge in three areas: (*i*) characterization of mitochondrial structure and function, (*ii*) elucidation of the steps involved in mitochondrial biosynthesis, and (*iii*) discovery of specific mitochondrial DNA. Many mitochondrial diseases are accompanied by mutations in this DNA. Inheritance is by maternal transmission. The metabolic defects encompass the electron transport complexes, intermediates of the tricarboxylic acid cycle, and substrate transport. The clinical manifestations are protean, most often involving skeletal muscle and the central nervous system. In addition to being a primary cause of disease, mitochondrial DNA mutations and impaired oxidation have now been found to occur as secondary phenomena in aging as well as in age-related degenerative diseases such as Parkinson, Alzheimer, and Huntington diseases, amyotrophic lateral sclerosis and cardiomyopathies, atherosclerosis, and diabetes mellitus. Manifestations of both the primary and secondary mitochondrial diseases are thought to result from the production of oxygen free radicals. With increased understanding of the mechanisms underlying the mitochondrial dysfunctions has come the beginnings of therapeutic strategies, based mostly on the administration of antioxidants, replacement of cofactors, and provision of nutrients. At the present accelerating pace of development of what may be called mitochondrial medicine, much more is likely to be achieved within the next few years.**

In 1959, the first biochemical studies of a cell organelle in humans were undertaken, following observations made at the bedside of a patient with striking symptoms, never before encountered. These clinical observations, first, led to an idea about the origin of the symptoms and, second, to studies of the particular organelle: the mitochondrion (1). The pathophysiology of the mitochondria developed gradually over the years as relevant discoveries were made in biochemistry, cell biology, and molecular biology. During the past few years the field *mitochondrial medicine* has expanded dramatically, in several directions. I here provide a short review concentrating on those aspects most relevant to clinical medicine. In the accompanying review (174), Wallace describes the molecular, biological, and evolutionary implications of mitochondrial diseases.

The Birth of Mitochondrial Medicine (1959–1962): Luft Disease

The first patient found to have a mitochondrial disease was a 30-year-old woman who developed clinical symptoms at the age of 7. Her dominant symptoms were *enormous perspiration* combined with markedly increased fluid intake but without polyuria; *extremely high caloric intake* (above 3000 kcal per day) at a stable body weight of 38 kg and a body height of 159 cm; and *general weakness*, particularly prominent in her musculature. The dominating laboratory finding was a basal metabolic rate (BMR, a measure of oxygen consumption) of +180%. Thyroid function was normal. Subtotal thyroidectomy with administration of thyroid-depressing drugs was followed by classical myxedema but with a BMR of +100%.

Following the idea that the patient's enormously elevated BMR must involve mechanisms regulating oxygen consumption at the cellular level, studies were undertaken that focused on the mitochondria of skeletal muscle. By 1960, studies on rat liver mitochondria had already shown that this organelle is site of cell respiration and respiration-regulated phosphorylation. Uptake of oxygen by mitochondria was known to be controlled by the components of ATP production (inorganic phosphate, P_i, and the phosphate acceptor, ADP). This respiratory control allows the body to adapt oxygen consumption to actual energy need. The patient's condition, *a priori*, could then be ascribed to a derangement of respiratory control.

Biochemical studies of isolated skeletal muscle mitochondria from the patient (Figs. 1 and 2) demonstrated a nearly maximal rate of respiration in the presence of substrate alone without addition of ADP + P_i, but an almost normal phosphorylating efficiency (expressed as the P/O ratio) in the presence of ADP and P_i. The mitochondria also exhibited high ATPase activity, which was only slightly stimulated by 2,4-dinitrophenol, a known uncoupler of respiration from phosphorylation. These features of "loosely coupled" respiration—deficient respiratory control with a partially maintained ability to synthesize ATP—accounted for the symptoms of the patient: abnormal production of heat, which the body tried to relieve by increased perspiration, and enormous caloric intake to compensate for the increased combustion.

The mitochondria in this patient were also insensitive to oligomycin, a drug which interferes with the tight coupling of electron transport to phosphorylation without inhibiting ATP synthesis. A tentative explanation for this observation was an "energy leak" above the level of the phosphorylating system. Such a proposal was supported by the observation in the second patient with this disease, Luft disease (2–4), of an energy-dissipating futile cycle of Ca^{2+} uptake and release—i.e., a waste of energy without a change in calcium concentration. Other remarkable findings in the first patient's mitochondria were a high level of cytochrome oxidase, a relatively low level of coenzyme Q, and a high content of RNA in a muscle homogenate, one piece of evidence for increased mitochondrial synthesis.

Electron microscopy (Fig. 3) of the mitochondria revealed striking structural abnormalities: large accumulations of mitochondria of highly variable size in the perinuclear zone of the muscle cells and vast paracrystalline inclusions, possibly composed of lipofuscin granules.

Several explanations for the loose coupling were tested, using techniques then available. These studies suggested that a short circuit of the flow of protons in the inner membranes had occurred, partly inhibiting ATP production—but preserving electron transport. No uncoupling agent—e.g., thermogenin (5)—was found in muscle homogenates.

Another assumption was that the lack of respiratory control might be due to en-

Abbreviations: KSS, Kearns–Sayre syndrome; MERRF, myoclonus epilepsy and ragged red fibers (syndrome); LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; CPEO, chronic progressive external ophthalmoplegia; CNS, central nervous system; LDL, low density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex.

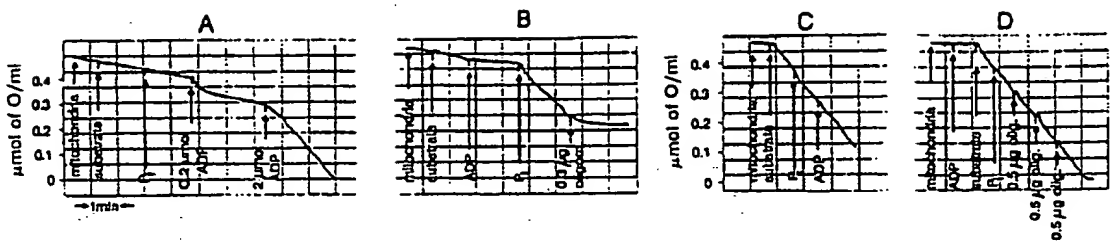

FIG. 1. Effect of P_i, ADP, and oligomycin on respiration of skeletal muscle mitochondria from a normal subject (*A* and *B*) and from the hypermetabolic patient (*C* and *D*).

hanced proliferation of mitochondria, with the formation of a component necessary for maintaining tight coupling between respiration and phosphorylation having failed to keep pace with the proliferation (6). Coenzyme Q might be a candidate for this—in the first patient its level was decreased relative to cytochrome oxidase.

The biochemical and morphological findings in Luft disease would have an impact on the further development of mitochondrial pathophysiology with the growth of the field in the 1970s.

Growth of the Field of Mitochondrial Disease

At the beginning of the 1970s it was realized that aberrations of the respiratory chain with or without structurally abnormal mitochondria of the type observed in Luft disease also occurred in certain other myopathies not associated with elevated oxygen consumption (7). In 1970–1972, respiratory chain deficiencies in disorders mainly involving central nervous system (CNS) and skeletal muscles were reported (8–10). In the following year, the first examples were reported of myopathies due to isolated deficiencies of muscle carnitine (11) and carnitine palmitoyltransferase (12).

These additional clinical discoveries were the starting point for rapid expansion in the field of mitochondrial pathophysiology. By 1988, Scholte's comprehensive review of the biochemical basis of mitochondrial diseases classified more than 120 entities (13). All were based on alterations in mitochondrial biochemistry.

From Scholte's and subsequent reviews several basic principles in mitochondrial pathology emerged. First, some mitochondrial diseases affect only one tissue, most often skeletal muscle and brain but also liver, heart, kidneys, or endocrine glands. Other organs may be involved secondarily. The disease may originate as a specific defect in mitochondrial function, but a variety of genetic and environmental factors may contribute to the phenotype.

Despite the diversity of clinical phenomena and mitochondrial pathology, seven syndromes have been particularly important in advancing our understanding of mitochondrial medicine: (*i*) *Kearns–Sayre syndrome* (KSS), with ophthalmoplegia, retinal pigmental degeneration, sometimes heart block, ataxia, hyperparathyroidism, and short stature; (*ii*) *myoclonus epilepsy and ragged red fibers syndrome* (MERRF), with intense myoclonus, epilepsy, progressive ataxia, muscle weakness and wasting, deafness, and dementia; (*iii*) *Leber hereditary optic neuropathy* (LHON), with blindness in men, at times movement disorders and encephalomyopathy, electrocardiogram abnormalities, and retinal microangiopathy; (*iv*) *mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes* (MELAS), with episodic vomiting, lactic acidosis, and myopathy with ragged red fibers, sometimes dementia, generalized seizures, deafness, and short stature; (*v*) *Leigh disease* or subacute necrotizing encephalomyopathy, with respiratory abnormalities, weak cry, impaired feeding, impaired vision and hearing, ataxia, weakness, and hypotension; (*vi*) *chronic progressive external ophthalmoplegia* (CPEO) and mitochondrial myopathy, with symptoms similar to those in KSS but also ocular myopathy, retinitis pigmentosa, and central nervous system (CNS) dysfunction; and (*vii*) *Alper syndrome* or progressive infantile poliodystrophy, with seizures, dementia, spasticity, blindness, and liver dysfunction accompanied by specific cerebral degeneration.

The clinical expressions of these and other mitochondrial syndromes may vary considerably. Overlapping between the syndromes is common and may make diagnosis difficult. Clearly, tissues with a high demand for ATP and oxidative turnover are preferentially affected in different combinations. In some syndromes, endocrine glands are involved with signs of diabetes, hypoparathyroidism, stunted growth, etc. Inherited factors are present in some, if not all, of these syndromes. Maternal inheritance is well established in MERRF and LHON.

A common feature in this group of inborn metabolic errors is the involvement of specific enzymes in the pathway of aerobic energy production in the mitochondria. Thus, there is a defect in coenzyme Q metabolism in KSS; reduced activities of respiratory complexes I and IV in MERRF; reduced activity in complex I in LHON; reduced activities in complex I and cytochrome *c* oxidase in MELAS; and reduced activity in cytochrome *c* oxidase in Leigh syndrome.

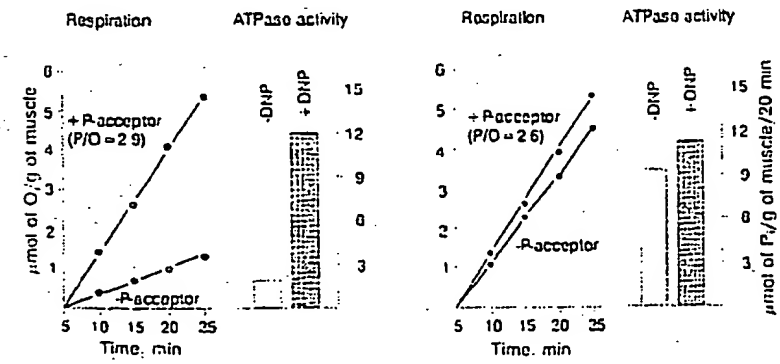

FIG. 2. Respiratory control, phosphorylation efficiency, and ATPase activity of isolated skeletal muscle mitochondria from a normal subject (*Left*) and from the hypermetabolic patient (*Right*). DNP, 2,4-dinitrophenol.

The Discovery of Specific Mitochondrial DNA (1963–1964) and of Mutations in It (1988)

That DNA is present in mitochondria (mtDNA) was first clearly shown in 1963 by Nass and Nass (14) in chick embryos and by Schatz *et al.* (15), who isolated DNA from purified yeast mitochondria. By 1981 the complete sequence of human

FIG. 3. Electron micrograph from a muscle fiber of the hypermetabolic patient. Cell nucleus (n) and a multitude of mitochondria (m) surrounding it. On the right is a bundle of dense cell incusions. (×4700.)

mtDNA was elucidated (16). Unlike nuclear DNA, there are thousands of copies of mtDNA in every nucleated cell, each mitochondrion containing 2–10 copies. In normal individuals, these copies are identical, each containing the genes encoding 13 proteins, all of which are subunits of the respiratory chain enzyme complexes, 22 tRNAs, and 2 rRNAs (for review, see refs. 17 and 18). The absence of introns makes mtDNA compact. The only noncoding part of mtDNA is the D loop (displacement loop) of about 1000 bp, containing the origin of replication of the H strand (heavy strand) of the mtDNA and the promoters for L- (light strand) and H-strand transcription (19). One of the fascinating features of mtDNA is that it undergoes mutations 5–10 times faster than nuclear DNA (20). This increased rate of mutation is due to at least two factors. (*i*) There is a lack of histone proteins to protect mtDNA (21). (*ii*) The mitochondria are not efficient in repairing DNA damage (22). About 90% of oxygen in the cell is consumed by mitochondria and, as a result, there can be extensive oxidative damage to mtDNA (23).

Mitochondria are the only known source of extranuclear DNA in humans. Since, during egg fertilization, the sperm contributes only its nuclear DNA to the zygote (19), the entire mitochondrial genotype in both males and females is maternally inherited. Thus, only the mothers transmit mtDNA to the children, and only the daughters can transmit mtDNA to the next generation. This inheritance does not follow Mendelian laws.

In 1988, a breakthrough in mitochondrial pathophysiology occurred with the report of an association of different sporadic human encephalomyopathies with large deletions of mtDNA (24) and a G-to-A transition mutation at nucleotide pair 11778 in the mtDNA of patients with LHON (25). A constant feature in LHON has been the coexistence of mutant and wild-type mtDNA (heteroplasmy). Following these reports, other clinical syndromes were soon linked to specific mutations, deletions, and duplications of mtDNA, impairing protein synthesis of the mitochondrial subunits of the respiratory chain complexes—e.g., point mutations of the $tRNA^{Lys}$ gene in the MERRF syndrome (26) and a point mutation of the $tRNA^{Leu}_{UUR}$ gene in MELAS (27). Most of the "classical" mitochondrial disorders have since been submitted to detailed studies (for reviews, see refs. 28–31). A special feature of these tRNA mutations is that they have indistinguishable consequences at the biochemical level, producing partial defects in the mtDNA-dependent respiratory complexes. In sporadic adult-onset CPEO with ragged red fibers, large-scale deletions ranging from 1.3 to 7.6 kb (32–35) or duplications (36) were observed in about 50% of the patients, and in nearly 100% of patients with KSS. No other mitochondrial encephalomyopathies had deletions (37). In some instances, evolution from a tissue-specific to a multisystem disorder (KSS) could be observed, and probably could be explained by an increase in the mutated mtDNA fraction with age (38).

In earlier studies, nuclear rather than mitochondrial mutations were thought responsible for the above defects. Rather, variation in the ratio of wild-type to mutant mtDNA in different tissues probably explains the tissue specificity of the mitochondrial myopathies (39). Similarly, alterations in the tissue distribution of the proportion of mutant mtDNA with time may explain some of the age dependency. However, disturbances in interactions between nucleus and mitochondria were recently reported in families with mitochondrial disease (CPEO-like syndrome) with autosomal dominant inheritance (for review, see ref. 31). The activities of respiratory complexes I and IV were markedly reduced, and there were multiple deletions of mtDNA spanning several kilobases. This autosomal dominant disease implied mutation in a nucleus-encoded gene (31). The abnormal product of this nuclear gene was supposed to interact with mtDNA to cause accumulation of multiple lesions in the molecule. This particular area was recently enriched by similar reports on CPEO syndromes and multiple deletions of mtDNA with autosomal recessive and autosomal dominant transmission (39–42). The next step towards better understanding the pathogenesis of these diseases must include studies of nuclear gene products interfering with mtDNA or its gene products. AIDS patients undergoing long-term treatment with azidothymidine (AZT) developed destructive mitochondrial myopathy with ragged red fibers and markedly reduced amounts of mtDNA in skeletal muscle, and this depletion was reverted in one patient after withdrawal of AZT (43).

Free Radicals, Oxidative Damage, and Antioxidants

Essential for the discussion of mitochondrial pathophysiology is a brief summary of oxidative processes in mitochondria and the consequences of abnormalities in those processes. Molecular oxygen has the ability to take up electrons (e^-) from the surroundings, and these electrons are easily exchangeable. During normal aerobic respiration, mitochondria consume O_2, reducing it stepwise to form H_2O (Fig. 4).

During this process, four electrons are added, and energy released is conserved as ATP. The chemical oxidants, $\cdot O_2$ and $\cdot OH$, are normal products of the oxidative process. Entities with such unpaired electrons and with reactive properties are called *radicals*. They may be harmful when produced in increased amounts and not neutralized by the normally occurring antioxidants. Then leakage may lead to damage of membrane lipids, DNA, proteins, and other macromolecules.

Other sources of radicals are destruction of cells during chronic infections (44) with bursts of NO, $\cdot O_2$, H_2O_2, and OCl^-; degradation of fatty acid and other molecules by peroxisomes (45); and by-products of processes acting as defense mechanisms against toxic substances (44). Exogenous additions to such endogenous contributors to the load of oxidants are, e.g., oxides of nitrogen (NO) in cigarette smoke, generation of radicals from peroxides promoted by iron and copper compounds (Fenton reaction), and products from normal food intake (46, 47). As a matter of fact, the effects of some anticancer drugs are based on this principle. Thus, Adriamycin targets cancer by producing reactive oxygen species.

Defense mechanisms try to minimize the levels of harmful oxidants and the damage they inflict. Several enzymes

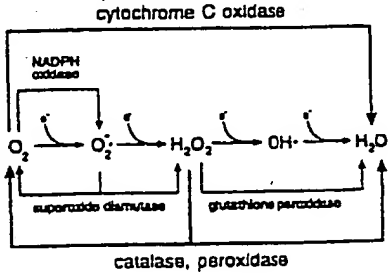

FIG. 4 Cellular formation of free radicals.

such as superoxide dismutase, catalase, and glutathione peroxidase are part of these mechanisms. The body also has developed natural lipophilic and hydrophobic antioxidants: vitamin E, the quinones (coenzyme Q), and carotenoids, typically located in membranes and in lipoproteins. Water-soluble antioxidants include vitamin C and thiols such as glutathione. Many of these antioxidants also are dietary products. The significance of vitamins Q and E as antioxidants has gained enormous attention during the last few years, especially coenzyme Q, located as it is in the electron transport system by linking complexes I and III of the respiratory chain. In its reduced form it serves as an antioxidant, preventing lipid peroxidation in biological membranes and low density lipoproteins (LDL) and, thereby, playing an active role in cellular defense against oxidative damage. Accordingly, coenzyme Q has been given to patients suffering from mitochondrial diseases.

The Aging Process and the Mitochondria

Many diseases related to aging may involve oxygen radicals at some stage in their development. In these diseases, it has been proposed that mutations of mtDNA and changes in cellular bioenergetics contribute in some way to the aging process and to the development of degenerative diseases. Thus, the capacity for oxidative phosphorylation declines with age (29) due to accumulation of defective mtDNA, nuclear DNA, or both. ATP production can decline below a level critical for the function of the cell. There is evidence that the "normal" aging process is accompanied by damage of molecules, including mtDNA, and that such damage accumulates with age (48–51). A 5-kb common deletion of mtDNA was found to accumulate with age in human brain and skeletal muscle (52). Furthermore, diseases associated with alterations in mtDNA progress with age, and this progression is associated with an increasing proportion of deleted molecules. In heart muscle, mtDNA deletions—especially a 3.6-kb deletion—have been shown to accumulate after 35 years of age (53–55), as has a 3-kb deletion in skeletal muscle (56). In addition, the concentration of mitochondrial mRNA and rRNA declined with age in rat brain and heart (57) and was associated with a 50% decrease in transcription rate (58). In ischemic hearts, hypoxemic inhibition of oxidative phosphorylation was accompanied by an increase in mtDNA damage (59).

As a sign of the aging process, the number of cytochrome *c* oxidase-negative skeletal and heart muscle fibers increased with age (60, 61), and enzyme activities of complexes I and IV declined progressively with age in human skeletal muscle and liver (62, 63). In addition, evidence has been presented for age-related changes in coenzyme Q levels in several tissue: by Beyer *et al.* (64) in rats, and by Kalén *et al.* (65) in humans.

While more substantial studies are needed, these data—concerning mtDNA, oxidation, and coenzyme Q—seem to favor the idea that aging may be associated with a disturbed balance between oxidative and antioxidative forces, leading to a decline in oxidative phosphorylation below some "organ-specific threshold" and to mtDNA damage. A morphologic consequence could be the age-related increase in lipofuscin granules, also termed "age pigments" (66).

Age-Related Degenerative Diseases and Defects in Oxidative Phosphorylation

In a search for diseases possibly connected with defects in oxidative phosphorylation and with alterations in mtDNA, it seems appropriate to look at tissues that are critically dependent on a large supply of ATP for their specific functions—e.g., CNS, heart and skeletal muscle, kidney, liver, retina, and pancreatic islets.

In this connection, some degenerative disorders—such as Parkinson disease and cardiomyopathies—are associated with deletions of the mitochondrial genome, in contrast to the classical encephalomyopathies (e.g., MERRF, MELAS, and LHON), characterized by distinct mutations. Ozawa *et al.* (67) recently expanded this knowledge by demonstrating that patients with degenerative disorders (Parkinson disease and dilated or hypertrophic cardiomyopathy) and classical encephalomyopathies (MERRF and MELAS), which carry some distinct but partly overlapping symptoms and pathologies, also carry similar clustering of point mutations of mtDNA. They emphasize, first, that these patients, while having phenotypically different disorders, belong to the same mtDNA gene family and, second, that not one particular mutation but the type and total number of mutations of a patient is an important factor for the expression of the disease. Brown *et al.* (68) supported this suggestion by reporting on synergistically interacting mutations of mtDNA in LHON, indicating that the clinical manifestations of the disease are the product of an overall decrease in mitochondrial energy production rather than a defect in a specific mitochondrial enzyme.

The Cardiovascular System. mtDNA deletions and depressed activities of the enzymes in oxidative phosphorylation in aging heart muscle could pave the way for a disturbed balance of oxidation/antioxidation. The imbalance could lead to free radical-mediated lipid peroxidation, including that of LDL. The aldehyde products of lipid hydroperoxide breakdown are responsible for the modification of LDL apoprotein. Aldehyde-modified apolipoprotein B alters receptor affinity and, therefore, is subjected to endocytosis via the scavenger receptor pathway of macrophages (69) and accumulates (70, 71). This accumulation can initiate foam cell formation and the appearance of atherosclerotic plaques (72–74). The oxidation of LDL may be prevented by endogenous antioxidant compounds, mainly α-tocopherol and coenzyme Q (75).

The approach to reduce such an assumed imbalance has been based on the general theme that defective energy supply—due to lack of substrate and cofactor and decreased utilization of oxygen—may lead to the progression of various myocardial diseases. This approach is supported by the findings that low levels of vitamins C and E, two of the natural plasma antioxidants, may contribute to the high incidence of ischemic heart disease (76, 77) and that cardiovascular diseases are accompanied by low plasma concentrations of vitamin C, α-tocopherol and β-carotene (78, 79), and coenzyme Q (80). The latter appears to protect human LDL more efficiently against lipid peroxidation than does α-tocopherol (81), demonstrating that LDL-associated coenzyme Q may be an important antirisk factor. Again, these studies must be considered preliminary.

Dilated Cardiomyopathy. Dilated cardiomyopathy is the most common cause of severe cardiomyopathies in young and middle-aged people. Deletions in mtDNA in heart muscle from such patients have been demonstrated (82, 83). Most of these cardial myopathies are familial (84), and in some families an X-chromosomal inheritance has been suggested (85). Interestingly, in a patient with familial dilated cardiomyopathy (86) mtDNA with deletions of different sizes made up about 50% of total mtDNA in heart muscle. This suggests the presence of some nuclear gene defect leading to multiple mtDNA deletions (see above).

Recently, a rapidly escalating number of reports have appeared on attempts to treat "cardiomyopathy" with compounds with antioxidant action, in particular coenzyme Q (87–92). While some of the reports may be promising, such treatment is yet to be established.

The CNS. The CNS derives its energy almost exclusively from oxidative phosphorylation and thus consumes a large amount of oxygen. Hydrogen peroxide (H_2O_2) is a normal by-product of the function of several enzymes of importance for the CNS—e.g., monoamine oxidase and tyrosine hydroxylase—and of the autooxidation of several endogenous substances (ascorbic acid and catecholamines). Any disturbances in the equilibrium between oxidation and antioxida-

tion in the CNS tissues could disrupt the efficiency of electron transport. This could decrease ATP availability to cellular functions in the CNS such as ATP-regulated K channels, Ca^{2+} pumps, Na^+/K^+ pumps, exocytosis, and various phosphorylation processes.

Another possible process leading to oxidative stress in the CNS could involve the major excitatory neurotransmitter glutamate (for review, see ref. 93) (Fig. 5). There is increasing evidence that glutamate may be a major mediator of oxidative stress in the CNS, primarily through its activation of ionotropic receptors, distinguished by specific agonists. Activation of glutamate receptors by these agonists in tissue culture leads to neuronal degeneration (94, 95). The processes include receptor-mediated influx of Na^+ and Ca^{2+}, which brings about a series of events including initiation of the arachidonic acid cascade, activation of proteases, and stimulation of NO synthase. The depolarization increases ATP consumption induced by Na^+/K^+ ATPase, increasing oxidative phosphorylation with superoxide radicals as a by-product. These radicals, as well as arachidonic acid, enhance the release of glutamate and inhibit its inactivation, thereby promoting the harmful events (96, 97). Furthermore, NO released in the above process interferes with many events, including oxidative phosphorylation, with a reduction in ribonucleotide reductase activity (98) and with formation of ·OH from O_2 (99), ultimately leading to degeneration of neurons (100).

Thus, stimulation of receptors for neurotransmitters—as in this case glutamate—may activate processes in the CNS leading to an imbalance in oxidation/antioxidation. This in turn could be accompanied by cumulative damage to DNA, proteins, and lipids, and eventually to degeneration of neurons. The results would be especially harmful if, for some reason, antioxidant defenses are compromised, as during aging.

However, systemic treatment with a variety of free radical scavengers did not protect against striatal lesions produced by intracerebral injection of glutamate receptor agonists (101, 102).

Neurodegenerative Diseases. Oxidative stress, perhaps partly glutamate mediated, has also been implicated in some neurodegenerative diseases. In *Parkinson disease* there is degeneration of dopaminergic neurons projecting into the caudate–putamen. The dopaminergic system may be at risk for oxidative stress (103), since the oxidation of catecholamines by monoamine oxidase, which increases with age, is a source of oxygen radicals (104). Enzyme assays in brains from Parkinson patients did reveal a reduction of complex I activity, especially in the substantia nigra (105–107), also observed in blood platelets (108) and skeletal muscle mitochondria (109, 110). Furthermore, some of the mtDNA-encoded subunits of complex I were decreased in the nigrostriatal region of brains from Parkinson patients (95). The amount of deletion-bearing relative to normal mtDNA in such patients was about 10 times larger than in controls (111). Parkinson disease has been suggested to appear when the genomes that have undergone deletions surpass a certain threshold, or the deletions are concentrated to a specific neuronal subtype of the striatum (31).

In addition, Parkinsonism is induced by a specific toxin of the substantia nigra, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a strong inhibitor of NADH-coenzyme Q reductase (complex I) and generator of oxygen radicals (112–114). Treatment with glutamate receptor agonists protected against the dopaminergic degeneration induced by an MPTP metabolite (115, 116). These data suggest a possible link between oxidative stress and glutamate neurotransmission in this system (93). For a while, emphasis was put on a 5-kb deletion of mtDNA in the striatum (111), but that could not be confirmed (115–117).

Amyotrophic lateral sclerosis (ALS). ALS is accompanied by progressive degeneration of motor neurons in the brain stem and spinal cord. In about 10% of the patients, ALS is inherited as an autosomal dominant trait with high penetrance after the sixth decade (118, 119). There are some data suggesting that oxidative stress and activation of glutamate-gated cation channels may be involved in ALS (93). Eleven different missense mutations in the gene encoding one form of cytosolic superoxide dismutase (SOD1)—responsible for the degradation of the toxic superoxide anion O_2^- to O_2 and H_2O—were observed in families suffering from the autosomal dominant form of ALS (120). In addition, the content of protein carbonyl, a measure of protein oxidation, was elevated in patients with sporadic ALS as compared with controls—at least suggesting oxidative stress as a feature of ALS (93). These data, while limited, may carry some important implications for future therapy in ALS (121).

Huntington disease (HD). HD is an autosomal inherited disorder, characterized by disturbances in movement and progressive dementia and with onset at a mature age. Intrastriatal injection of a glutamate receptor agonist reproduced several aspects of the neuropathology of HD, indicating some dysfunction in the disposition of excitatory amino acids (122). The levels of glutamate in cerebrospinal fluid were reported to be elevated in HD (123). Pharmacological inhibition of complex I or complex II caused the same selective pattern of degeneration as seen with glutamate receptor agonists (124, 125). Neuronal susceptibility to complex II inhibition increases with age in animals, which may be germane to the

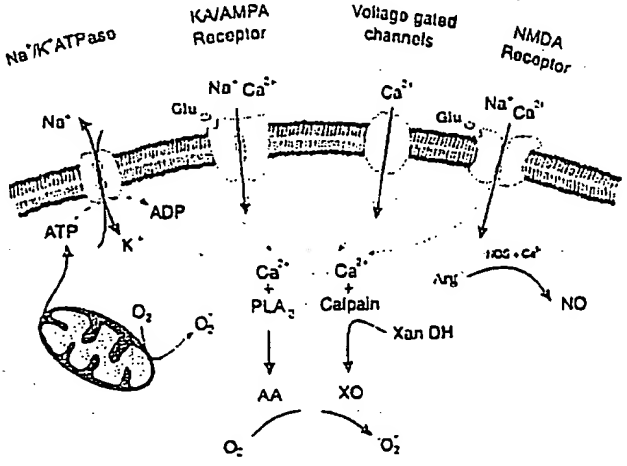

FIG. 5. Schematic representation of the glutamate receptor-mediated processes that may be involved in the generation of oxidative stress. This is a modification of the model presented by Coyle and Puttfarcken (93). Glutamate activates the kainic acid/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (KA/AMPA) receptor, which results in opening of channels through which Na^+ and Ca^{2+} flow. Depolarization activates voltage-gated Ca^{2+} channels, enabling Ca^{2+} influx and thereby increasing cytoplasmatic free Ca^{2+}, $[Ca^{2+}]_i$. Under partially depolarizing conditions, Na^+ and Ca^{2+} flow through channels of the *N*-methyl-D-aspartate (NMDA) receptor. An increase in $[Ca^{2+}]_i$ may activate various enzymes such as phospholipase A_2 (PLA_2), proteases, and NO synthase (NOS), promoting the formation of $\cdot OH$, O_2^- and NO. $\cdot OH$, O_2^- is also produced as a by-product in the increased oxidative phosphorylation, which follows an increased ATP consumption by the Na^+/K^+ ATPase. AA, arachidonic acid; Xan DH, xanthine dehydrogenase; XO, xanthine oxidase

delayed onset of neurodegeneration in HD (126). The ensuing disruption of the respiratory chain could lead to impaired oxidative phosphorylation. In addition, abnormal mitochondrial structures and accumulation of lipofuscin have been demonstrated in HD (127). A complex IV defect in the caudate, but not in the cortex, was found in brains from patients with HD (128), and also a complex I defect in blood platelet mitochondria (129). These and other data favor the possible involvement in HD of oxidative stress, perhaps in combination with dysfunction of glutamate metabolism.

Alzheimer disease. Alzheimer disease is an age-related dementia, characterized pathologically by neurofibrillary tangles, senile plaques, and amyloid deposits in the CNS. There are indications for defects in mitochondrial function in this disease: oxidative phosphorylation was not effectively coupled in homogenates of neocortex from patients (130); there were marked reductions in pyruvate dehydrogenase in frontal and occipital cortex (131) and in complex I activity in blood platelet mitochondria from patients (132); and distinct point mutations of mtDNA were reported in brain sections (133). Therefore, the development of Alzheimer disease to some extent involves components of mitochondrial energy production, including degeneration of synaptosomes because of impaired energy production of synaptosomal mitochondria (29).

On the whole, oxidative stress seems to represent one possible pathway—perhaps in part initiated by glutamate—leading to neuronal degeneration in a manner consistent with the course and pathology of some degenerative diseases of the CNS. However, antioxidants at best provided only partial protection, and oxidants can be generated by a number of mediators independent of glutamate (134). Furthermore, other pathologic processes may be the primary events enhancing the vulnerability to glutamate, such as the amyloid A4 peptide in Alzheimer disease (135). These and other observations and views demonstrate the gaps in our knowledge of the specific metabolic processes that may promote oxidative stress at the neuronal level, including glutamate receptor activation (93). Filling these gaps may lead to strategies for blocking pathways involved in neuronal degeneration.

Diabetes Mellitus

Non-insulin-dependent diabetes mellitus (NIDDM) is an age-related disease, which also exhibits features of a degenerative disorder. Can an increase in the incidence of insulin-dependent diabetes mellitus (IDDM) be on the basis of environmental exposure or other factors involving the mitochondrial genome? There are several lines of evidence suggesting alterations in mtDNA in two of the major tissues involved in diabetes, pancreatic islets and skeletal muscle, both highly reliant on oxygen. Formation of free radicals such as NO and ·OH and alkylation of DNA and proteins also occur in the beta cells of the pancreatic islets, and the inadequately protected mitochondrial genome is open to attack from such chemicals. Various diabetogenic agents could operate by this route—e.g., interleukin 1β, interferon γ, tumor necrosis factor α, alloxan, and streptozotocin (ref. 136; for review, see ref. 137). The action of some of these agents could be inhibited by antioxidants in animal models (138–141) as well as in humans (142). Such observations led Okamoto (143) to suggest that diabetogenic agents induce breaks of mtDNA in islets, ultimately followed by death of beta cells. Universal applicability of this hypothesis has been questioned (144).

Gerbitz (137) discusses whether mitochondrially encoded peptides can serve as MHC-restricted antigens. Some findings point in this direction (145, 146). If future research verifies this possibility, autoreactivity would enter the scheme leading to IDDM.

There is already some clinical evidence for the involvement of mtDNA in the development of diabetes. Patients with KSS and CPEO (see above) have an incidence of diabetes several times higher than in the general population (147–149). The earlier the onset of mitochondrial myopathy in these conditions, the more frequent was its association with IDDM (150). Also, MELAS and other mitochondrial cytopathies are sometimes associated with diabetes (151) and with a point mutation of mtDNA (152). This makes it likely that alterations of mtDNA of the beta cells may contribute to the development of diabetes.

Recently, a systemic 10.4-kb mtDNA deletion was reported in a family with maternally transmitted diabetes and sensor-neural deafness (153). Subsequently an A-to-G transition at nucleotide pair 3243, a conserved position in the mitochondrial gene for $tRNA^{Leu}_{UUR}$, also has been reported in families with diabetes (154–156). This mutation leads to impairment of mitochondrial transcription termination, which causes defects in mitochondrial protein synthesis (157). A similar mutation was found in insulin antibody-positive subjects, initially diagnosed as NIDDM, who progressed to IDDM (158). The mutation may be connected with a variable and progressive decrease in insulin secretory capacity.

It is unlikely that "common" NIDDM, constituting about 90% of the diabetic population, has its origin in specific mutations of mtDNA. However, an age-related decline in the capacity for oxidative phosphorylation and its consequences could play a significant role in its pathophysiology.

Therapeutic Aspects

Understanding the mechanisms behind the development of mitochondrial diseases offers strategies for attempts at their treatment. Possibilities are supplementation of cofactors in the respiratory chain, addition of oxidizable substrates, and prevention of oxygen radical damage to the mitochondria.

Favorable results with antioxidants, "redox therapy," were reported in a patient with a severe defect in complex III of the respiratory chain (159). Favorable results have also been reported in other circumstances (for reviews, see refs. 160 and 161): e.g., with coenzyme Q and succinate in a patient with KSS and a complex I defect (162) and with a prominent complex IV lesion (163); with coenzyme Q in ocular myopathy (164); and with coenzyme Q in five patients with KSS and low levels of coenzyme Q in serum and the mitochondrial fraction of skeletal muscle (164). Coenzyme Q also occupies a special place in the attempts to normalize oxidation/antioxidation abnormalities in age-related disease. Again, however, many of the reports on the use of coenzyme Q are anecdotal and require substantiation.

The rationale for treatment of cardiomyopathy with coenzyme Q rests, in part, on the finding of myocardial dysfunction and defective energy supply in biopsy samples from subjects with such pathology (165). While there are favorable reports on administration of coenzyme Q in that circumstance (166–169), additional studies are required to establish its possible role in that therapy.

Other ways of treating diseases that can be attributed to dysfunction of oxidative phosphorylation have been tried (170–173). One in LHON includes functional relocation of normal mitochondrial genes to the patient's nucleus so that their protein products are delivered to the organelle from the cytoplasm. In another, myoblasts from patients with mitochondrial myopathy have been explanted, and their mutant DNA has been replaced with normal DNA. The genotypically normal muscle cells have then been expanded and injected back into the patient's muscle, where they could fuse to existing myotubes, contributing more normal mtDNA and supplementing mitochondrial energy production.

Conclusions

We can anticipate expansion of the field of mitochondrial medicine in several directions: first, into some age-related diseases so far not approached; second, into

areas connected with new concepts in mitochondrial biochemistry and physiology—e.g., protein transport via the mitochondrial membrane. Of great interest are in-depth studies aiming at a better understanding of the processes underlying the mitochondrial defects and well-controlled studies on the place of nutritional supplements and replacement therapy with suitable redox compounds in the amelioration of disorders due to deficiencies in mitochondrial bioenergetics.

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Mitochondrial dysfunction in neurodegenerative diseases

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Abstract

A potential pivotal role for mitochondrial dysfunction in neurodegenerative diseases is gaining increasing acceptance. Mitochondrial dysfunction leads to a number of deleterious consequences including impaired calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition and secondary excitotoxicity. Neurodegenerative diseases of widely disparate genetic etiologies may share mitochondrial dysfunction as a final common pathway. Recent studies using cybrid cell lines suggest that sporadic Alzheimer's disease is associated with a deficiency of cytochrome oxidase. Friedreich's ataxia is caused by an expanded GAA repeat resulting in dysfunction of frataxin, a nuclear encoded mitochondrial protein involved in mitochondrial iron transport. This results in increased mitochondrial iron and oxidative damage. Familial amyotrophic lateral sclerosis is associated with point mutations in superoxide dismutase, which may lead to increased generation of free radicals and thereby contribute to mitochondrial dysfunction. Huntington's disease (HD) is caused by an expanded CAG repeat in an unknown protein termed huntingtin. The means by which this leads to energy impairment is unclear, however studies in both HD patients and a transgenic mouse model show evidence of bioenergetic defects. Mitochondrial dysfunction leads to oxidative damage which is well documented in several neurodegenerative diseases. Therapeutic approaches include methods to buffer intracellular ATP and to scavenge free radicals.

Keywords: Mitochondrion; Huntington's disease; Friedreich's ataxia; Alzheimer's disease; Amyotrophic lateral sclerosis; Oxidative damage

1. Introduction

Neurodegenerative diseases are a heterogeneous group of illnesses with distinct clinical phenotypes and genetic etiologies. Major advances in understanding the pathogenesis of these illnesses have come from molecular genetics. Despite the presence of genetic defects in widely varying proteins substantial evidence points to mitochondrial dysfunction as a unifying fundamental mechanism involved in neuronal degeneration. Mitochondrial dysfunction has widespread deleterious ramifications for cellular function which are discussed in greater detail in other subsections of this review. In brief mitochondrial dysfunction leads to impaired energy production, impaired cellular calcium buffering, activation of proteases and phospholipases, activation of nitric oxide synthase, and generation of free radicals. The above pathways can lead to either apoptotic or necrotic cell death depending on the severity of the insult.

The present review will focus on four prototypical neurodegenerative diseases which are associated with

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either nuclear or mitochondrial DNA mutations which either directly or indirectly lead to mitochondrial dysfunction. Recent evidence implicates a cytochrome oxidase deficiency in Alzheimer's disease. There is a mutation in a nuclear encoded mitochondrial protein in Friedreich's ataxia. In familial amyotrophic lateral sclerosis mitochondrial dysfunction may be a consequence of oxidative damage due to point mutations in Cu,Zn superoxide dismutase. In Huntington's disease the means by which the nuclear DNA encoded expansion of CAG repeats in huntingtin results in mitochondrial dysfunction remain to be clarified, yet increasing evidence implicates an impairment of bioenergetics in Huntington pathogenesis.

2. Alzheimer's disease

Alzheimer's disease (AD) is the most common of the neurodegenerative diseases. The most important risk factor is advancing age [1]. The illness occurs in both a familial form which is autosomal dominant inherited and an apparently sporadic illness. Familial autosomal dominant Alzheimer's disease is associated with point mutations in the amyloid precursor protein as well as in novel proteins entitled presenilin-1 and presenilin-2. Familial Alzheimer's disease accounts for approximately 5% of all cases. The remaining apparently sporadic cases of Alzheimer's disease show an increased risk in families of 2.5–3-fold. A sporadic inheritance pattern with familial association and evidence for maternal transmission are characteristic features of known mitochondrial genetic diseases. Some evidence has suggested that there is maternal inheritance in Alzheimer's disease [2,3]. These studies showed an increase in female to male ratio in the parental generation of Alzheimer's disease probands. In a group of families in which there were both an affected parent and at least two affected siblings the ratio of mothers to fathers in the parental generation was 9:1 [2].

There is substantial evidence implicating metabolic defects in Alzheimer's disease. Studies utilizing positron emission tomography consistently show reduced glucose metabolism in temporoparietal regions of Alzheimer's disease patients, and this appears to occur quite early in the disease course [4]. Recent studies have demonstrated that this occurs in patients at risk for Alzheimer's disease [5], and there appeared to be reduced glucose utilization in asymptomatic patients who are homozygous for the Apo ε4 allele, a known risk factor for sporadic Alzheimer's disease [6]. Positron emission tomography studies also show increased oxygen utilization in comparison with glucose utilization in Alzheimer's disease patients [7]. This latter observation has been confirmed with direct measurements in arterial and jugular venous samples [8,9]. Prior work also demonstrated abnormal glucose metabolism in brain biopsy specimens [10]. Phosphorus magnetic resonance spectroscopy has demonstrated abnormalities in either phosphocreatine (PCr) or inorganic phosphate (Pi) in Alzheimer's disease patients as compared with elderly controls [11,12]. The study of Smith and colleagues demonstrated a reduction in PCr/Pi ratio in the frontal cortex of Alzheimer's disease patients [13].

Initial studies which suggested that there were defects in cytochrome oxidase in Alzheimer's disease were done in platelets. Parker and colleagues reported significant decreases in cytochrome oxidase activity in Alzheimer's disease platelets as compared to normal controls [14]. This work was disputed utilizing less purified platelets preparations but was confirmed in a follow-up study [15,16]. Studies of postmortem cerebral tissue of Alzheimer's disease patients confirmed reduced cytochrome oxidase activity [17,18]. The cytochrome oxidase activity shows a reduction in catalytic activity yet normal amounts of cytochrome aa_3, suggesting that reduced complex IV activity is a consequence of abnormal catalytic activity rather than decreased enzyme levels [16].

Although prior biochemical studies suggested that there were decreases in cytochrome oxidase activity in Alzheimer's disease platelets and cerebral tissue, it was unclear whether this was a primary or secondary effect of the disease process. A novel technique to investigate the role mitochondrial defects is to utilize cybrid technology, which was pioneered by King and Attardi [19]. This technique involves the transfer of mitochondria from living patients or cell lines to mitochondria deficient cells (ρ^0 cells). Cell lines from a variety of sources can be depleted of mitochondrial DNA (mtDNA) by exposing them to low concentrations of ethidium bromide. Ethidium bromide is concentrated within mitochondria and preferentially in-

hibits mtDNA replication in comparison to nuclear DNA replication. Exposed cells lose their mtDNA and assume an anaerobic phenotype. Cybrids are cells formed by fusing mitochondria from platelets or other tissues into the ρ^0 cells. Since the ρ^0 cells are auxotrophic for uridine and pyruvate, any cells which are not transformed are then eliminated by removing uridine and pyruvate from the medium. The resulting cybrids then enable one to determine whether any observed defects in oxidative phosphorylation are attributable to alterations in the patient's mtDNA, since the patient's mitochondria now function in the presence of a different nuclear background.

Recent studies using the cybrid technique to demonstrated that the cytochrome oxidase defects in Alzheimer's disease appear to be encoded on mtDNA [20,21]. It was shown that cytochrome oxidase defects can be transferred from Alzheimer's disease platelets into cybrids Furthermore the ensuing cybrid cell lines show markedly increased free radical production. Point mutations were found in the cytochrome oxidase-1 and cytochrome oxidase-2 mtDNA encoded subunits of cytochrome oxidase, however further work needs to be done to exclude the possibility that these mutations are not present in nuclear pseudogenes. Nuclear pseudogenes are mitochondrial DNA sequences which are randomly incorporated into the nuclear genome by unclear mechanisms, but which exist for much of the mitochondrial genome.

The consequences of cytochrome oxidase defects in cybrid cell lines on intracellular calcium buffering have been determined [22]. The Alzheimer's disease cybrids show elevated basal cytosolic calcium concentrations as well as enhanced sensitivity to inositol-1,4,5-triphosphate mediated calcium release. They also show slower recovery from the increased calcium levels. These findings are consistent with prior observations in AD fibroblasts [23–25]. They are also consistent with the finding of decreased calcium uptake in mitochondria from AD fibroblasts [26]. Impaired calcium buffering is also known to occur in fibroblasts of patients with a known mitochondrial disorder, MELAS syndrome [27].

Alzheimer's disease cybrid cell lines are associated with increased free radical production. One therefore might expect that there would be evidence for increased free radical damage in Alzheimer's disease postmortem tissue. Mitochondrial DNA may be preferentially vulnerable since it is located close to the inner mitochondrial membrane. Consistent with this possibility we found a 3-fold increase in 8-hydroxy-2-deoxyguanosine content of mtDNA in AD postmortem tissue as compared to age-matched controls [28]. In other studies there have been reports of increased tissue concentrations of malondialdehyde and protein carbonyl groups [29,30]. Furthermore novel spin trapping techniques demonstrated increased oxidative damage to both lipids and proteins [31]. Immunocytochemical studies have demonstrated that there is evidence for oxidative damage at the cellular level. Neurofibrillary tangle bearing neurons showed increased immunostaining with antibodies to advanced glycation end products, hemeoxygenase-1, malondialdehyde, 4-hydroxynonenal, protein carbonyl groups, carbonylated neurofilaments, and 3-nitrotyrosine [13,32–38]. Interestingly these antibodies show increased staining in cell bodies and neurites rather than in senile plaques, suggesting an intracellular source of free radicals.

Mitochondrial dysfunction may be linked to the other neuropathological hallmarks of Alzheimer's disease including senile plaques and neurofibrillary tangles Previous studies showed that impairment of cytochrome oxidase in vitro leads to an increase in C-terminal fragments of the amyloid precursor protein, which contain the β-amyloid peptide [39], and a decrease in non-amyloidogenic processing of the amyloid precursor protein [40]. An increase in intracellular β-amyloid 1–42 was found after exposure of cultured guinea pig neurons to hydrogen peroxide, and oxidative stress increased β-amyloid in mammalian lens tissue [41,42]. An increase in intracellular β-amyloid was also observed in cultured astrocytes from Down syndrome patients in which there is increased free radical production [43]. Qxidative damage may lead to cross-linking and impaired solubility of β-amyloid [44,45].

Oxidative injury has also been shown to lead to intermolecular cross links in covalent bonds which could contribute to the generation of paired helical filaments [46]. Oxidation of critical cysteine residues seems to be associated with the aggregation of tau proteins into paired helical filaments [47] Reduced ATP generation also leads to activation of ERK1

and ERK2 kinases which phosphorylate tau proteins into a paired helical filament-like state similar to that in AD [48,49].

3. Friedreich's ataxia

A role of mitochondrial dysfunction in Friedreich's ataxia has been greatly strengthened by recent observations. Friedreich's ataxia is characterized by neurodegeneration involving the spinocerebellar pathways as well as a cardiomyopathy. The gene product was recently cloned and designated frataxin [50]. Frataxin deficiency may be a consequence of nonsense or missense point mutations, but the primary cause appears to be an expansion of a polymorphic GAA trinucleotide repeat situated in the first intron of the corresponding gene. This results in marked reduction in steady state levels of mature frataxin mRNA. There appears to be a correlation between the regions of degeneration observed in the disease and the sites of frataxin transcription, which are highest in the heart, spinal cord and dorsal root ganglia [51]. Recent studies show that a gene from yeast is homologous to the human frataxin protein [52]. This gene encodes a mitochondrial protein involved in iron homeostasis and respiration function. Human frataxin has been linked to green fluorescent protein and was shown to be localized to mitochondria [51–54]. Disruption of the yeast homologous gene results in respiratory insufficiency with an inability to carry out oxidative phosphorylation [51,52,54], as well as a loss of mitochondrial DNA [52,54]. Yeast with disruption of their frataxin homologue show marked increases in iron transport, increases in iron content, and hypersensitivity to oxidative stress mediated by H_2O_2 [52]. These observations suggest that impaired function of this protein most likely leads to mitochondrial dysfunction and results in hypersensitivity to oxidative stress, presumably mediated by iron catalyzed Fenton chemistry. This speculation is supported by studies of endomyocardial biopsies of patients with Friedreich's ataxia [55] The biopsies show deficiencies of aconitase and complexes I, II and III of the electron transport chains which contain iron-sulfur clusters and are known to be susceptible to oxidative stress. It is also of interest that a Friedreich's ataxia-like syndrome with retinitis pigmentosis is caused by mutations in the α-tocopherol transfer protein [56–58]. This results in reduced concentrations of vitamin E. Neurological symptoms in this disorder include ataxia, dysarthria, hyporeflexia and decreased proprioceptive sensation.

Other evidence implicating mitochondrial dysfunction in Friedreich's ataxia includes an increased incidence of diabetes mellitus and optic atrophy which are frequent complications of mitochondrial disorders. Positron emission tomography studies of Friedreich's ataxia patients have detected cerebral glucose hypermetabolism early in disease progression [59]. Biochemical studies found increased blood lactate levels and generally abnormal carbohydrate metabolism in patients with Friedreich's ataxia [60]. Reduced activities of several mitochondrial enzymes including α-ketoglutarate dehydrogenase and pyruvate dehydrogenase have also been reported [61].

4. Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a prototypical neurodegenerative disease of late life characterized by progressive muscle weakness, atrophy and spasticity [62]. It leads to paralysis and death within 3–5 years after onset. Characteristic neuropathologic features are loss of anterior horn motor neurons as well as degeneration of the corticospinal tracts. Ninety percent of ALS cases are apparently sporadic with no identifiable genetic or environmental risk factors. The remaining 10% of cases show familial autosomal dominant inheritance. A major advance in the understanding of ALS was the identification of point mutations in the enzyme superoxide dismutase in approximately 25% of patients who have familial ALS [63]. More than 60 mutations have now been associated with the disease. These mutations typically affect the protein backbone of the enzyme and may interfere with normal dimer interactions. Only two mutations have thus far been reported which may affect the active site copper.

The observation that mutations in superoxide dismutase cause familial ALS suggested that oxidative injury might be playing role in its pathogenesis. Although there is a decrease in superoxide dismutase activity in familial ALS patients, strong evidence sug-

gests that the genetic defect leads to a gain of function from the mutant enzyme. This evidence includes the dominant inheritance pattern of familial ALS, the lack of correlation between enzyme activity and disease severity [64], and the observation that overexpression of the mutant enzyme in transgenic mice leads to motor neuron degeneration [65–67]. The mutations appear to alter the stability of the protein backbone and reduce the half-life of the molecule [68]. They also appear to alter zinc binding [69]. These changes may relax the conformation of the active channel thereby allowing increased access of hydrogen peroxide or peroxynitrite to the active site copper. This is predicted to increase generation of hydroxyl radicals or nitronium ions which can then nitrate proteins [70]. In support of this two in vitro studies showed that superoxide dismutase with two different familial ALS mutations can generate increased amounts of hydroxyl radicals as compared to wild-type molecules [71–73]. Furthermore we recently obtained evidence in two different strains of transgenic ALS mice that there are increases in 3-nitrotyrosine concentrations, consistent with increased accessibility of peroxynitrite to the active site copper [74,75]. Of interest in both of these lines of transgenic ALS mice mitochondrial vacuolization and swelling are prominent pathologic features [65,67]. Furthermore we found increased complex I activity in both postmortem brain material of patients with the A4V superoxide dismutase mutation as well as in transgenic mice with the G93A superoxide dismutase mutation [76]. It is possible that increased generation of free radicals may damage the inner mitochondrial membrane. This could lead to a proton leak which would therefore have to be compensated by increased activity of the mitochondrial electron transport complexes responsible for proton transport. Expression of superoxide dismutase with the G93A mutation in neuroblastoma cells in vitro leads to a loss of mitochondrial membrane potential and increases in cytosolic calcium [77].

In sporadic ALS there is also evidence to suggest impairment of energy metabolism. Reduced glucose metabolism has been observed in cerebral cortex as sporadic ALS patients using positron emission tomography [78,79]. We recently examined electron transport enzymes in postmortem tissue of sporadic ALS patients. No alterations were found but this could be due to heterogeneity of defects amongst patients, which would obscure them due to an averaging effect [76]. Furthermore abnormalities could be obscured by astrogliosis. A recent study using the cybrid cell technique showed that there appeared to be mild decreases in complex I and complex IV activities associated with sporadic ALS [80]. Furthermore peripheral blood lymphocytes from sporadic ALS patients show increased cytosolic calcium and impaired responses to uncouplers of oxidative phosphorylation [81]. Studies of motor neurons in sporadic ALS patients show an accumulation of mitochondria in proximal axons, and muscle biopsies show increased mitochondrial volume and calcium levels [82–84]. Liver biopsies show enlarged mitochondria with intramitochondrial inclusions in sporadic ALS patients [85]. Mitochondria with abnormal protrusions were observed in anterior horn cells of a familial ALS patient who was later determined to have a superoxide dismutase mutation [86].

A consequence of mitochondrial dysfunction in either familial or sporadic ALS patients may be increased oxidative damage. Both we and others have observed increased protein carbonyl groups in the motor cortex and spinal cord of ALS patients [76,87]. We recently found increased concentrations of 3-nitrotyrosine and its major metabolite 3-nitro-4-hydroxyphenylacetic acid in the thoracic and lumbar spinal cord of both sporadic and familial ALS patients [88]. Immunocytochemical staining also demonstrated increased 3-nitrotyrosine in anterior horn cells.

A pathologic hallmark of familial and sporadic ALS is the accumulation of neurofilaments in proximal axons [89,90]. This may be a consequence of oxidative damage or it could be due to accumulation of 3-nitrotyrosine in the neurofilament light chain [70], which impairs normal aggregation of neurofilaments [91,92]. Furthermore an energy defect may contribute to slowing to axonal transport which has recently been demonstrated in sporadic ALS patients, in which there was an accumulation of mitochondria in proximal axons [83].

5. Huntington's disease

The genetic defect in Huntington's disease (HD)

consists of an expanded CAG repeat in a gene located on chromosome 4. The gene encodes a novel protein designated huntingtin which is widely distributed in both the peripheral tissues of the body as well as in the central nervous system. Both the normal function of huntingtin as well as its role in the pathogenesis of neuronal degeneration in Huntington's disease are obscure. Substantial evidence however suggests that the disease mutation leads to a gain of function causing cell type specific neuronal degeneration predominantly in the striatum. Evidence in support of this includes the observation that heterozygote knockout mice of the gene huntingtin do not show any phenotypic abnormalities [93]. The cleavage of huntingtin leads to N-terminal fragments which aggregate to form intranuclear inclusion bodies and aggregates in dystrophic neurites [94].

There is a substantial body of evidence implicating defects in energy metabolism in HD. Decreased glucose metabolism using positron emission tomography has been consistently shown in HD patients. The reductions in glucose metabolism occur in presymptomatic patients [29]. Furthermore we utilized proton magnetic resonance spectroscopy to demonstrate that there are elevated lactate concentrations in both the occipital cortex and in the basal ganglia of HD patients [95]. Increased lactate concentrations occur in the basal ganglia of some but not all presymptomatic patients, suggesting that the metabolic defects can precede clinical manifestations of the illness. Other authors found increased lactate in both frontal cortex as well as in occipital cortex and cerebellum [96,97]. We observed increased lactate concentrations in both parietal and supplementary motor cortex, suggesting that there is a widespread metabolic defect in Huntington's disease brain tissue. Furthermore we found a decrease in PCr/Pi ratio in resting gastrocnemius muscle of HD patients, providing evidence that there are defects in energy metabolism in peripheral tissues as well [98]. The latter observation is consistent with reports of progressive weight loss in HD patients despite increased caloric intake [99]. Furthermore we found that cerebrospinal fluid lactate to pyruvate ratios are increased in HD subjects as compared to age-matched controls, and similar observations have been made in patients with Machado-Joseph disease which is also due to an expansion of CAG repeats [98,100]

Reports of changes in enzymes involved in oxidative phosphorylation have shown reduced succinate dehydrogenase activity in HD postmortem brain tissue [101,102]. Reports of mitochondrial electron transport enzymes yielded inconsistent results. Two recent studies however demonstrated a 55–60% decrease in complex II–III activity in Huntington's disease basal ganglia [103,104]. Smaller decreases in cytochrome oxidase activity were less consistent. Complex I and citrate synthase activity showed no changes. The most consistent alterations in electron transport activity therefore appear to be decreases in complex II–III activity in the basal ganglia of HD patients. This is of interest since genetic defects affecting complex II–III activity are associated with basal ganglia degeneration [105], and comparable defects in experimental animals result in striatal degeneration [106]. Studies in cultured fibroblasts from HD and control patients also show mitochondrial defects [107]. Ionomycin induced calcium influxes result in depolarization of the mitochondrial membrane potential. Normal fibroblasts show depolarization followed by recovery, but Huntington's disease fibroblasts show failure to recovery fully after the second application of ionomycin.

One proposed mechanism by which the HD gene defect could lead to impaired energy metabolism is by an interaction between huntingtin and glyceraldehyde-3-phosphate dehydrogenase, a critical component of the glycolytic pathway [108]. It was suggested that an increase in polyglutamine repeats in huntingtin potentially inhibits the enzyme. Indeed we found that intrastriatal administration of the glyceraldehyde-3-phosphate dehydrogenase inhibitor iodoacetate produces dose dependent striatal lesions, which correlate with inhibition of enzyme activity [109]. However we could not confirm a decrease in glyceraldehyde-3-phosphate dehydrogenase activity in postmortem brain tissue of Huntington's disease patients [104], however a competitive inhibition could still occur. There is limited evidence for oxidative damage postmortem brain tissue of Huntington's disease patients. We found increased concentrations of 8-hydroxy-2-deoxyguanosine in nuclear DNA in Huntington's disease caudate [104]. Furthermore our initial immunocytochemical studies showed increased staining of striatal neurons of HD patients with antibodies to hemeoxygenase-1, 8-hydroxy-2-de-

oxyguanosine and malondialdehyde modified protein [110].

Other evidence implicating mitochondrial dysfunction in the pathogenesis of Huntington's disease comes from studies of mitochondrial toxins. We utilized 3-nitropropionic acid, an irreversible inhibitor of succinate dehydrogenase, to attempt to model Huntington's disease in both rodents and primates [106,111]. Accidental ingestion of 3-nitropropionic acid in man produces selective basal ganglia lesions and dystonia [112]. Systemic administration of 3-nitropropionic acid to nonhuman primates results in both a movement disorder as well as frontal type cognitive deficits which are similar to those which occur in HD [106,113]. Histological evaluation showed basal ganglia degeneration with sparing of NADPH diaphorase interneurons, dendritic abnormalities in spiny neurons and sparing of the nucleus accumbens, all of which are characteristic features of Huntington's disease neuropathology.

Further evidence for a metabolic defect in HD comes from a recent transgenic animal model [114]. Transgenic mice were made expressing a large number of CAG repeats (130–140) in exon 1 of the HD gene. These mice developed normally for about 8 weeks followed by onset of a movement disorder with tremors and subsequent seizures. The mice die at approximately 12–14 weeks of age. Intranuclear inclusion bodies which stain with N-terminal antibodies to huntingtin develop preceding the onset of symptoms [20]. It has therefore been suggested that these nuclear inclusion bodies may play a role in disease pathogenesis. Although there is no degeneration of the basal ganglia the brains appear to be small, and this occurs early in the disease process.

A characteristic feature of these mice is progressive weight loss despite increased caloric intake [114]. These findings are consistent with those observed in Huntington's disease patients who also appear to have increased caloric intake despite weight loss [99]. These findings therefore suggest that there may indeed be a metabolic defect in both a transgenic animal model of HD as well as in patients. Furthermore we have recently studied another transgenic animal model of HD in which there is a expansion of either 48 or 66 repeats in the full length huntingtin protein (Browne et al., unpublished findings). These mice as yet have not shown any phenotypic abnormalities. The mice however show increased 2-deoxyglucose uptake in the basal ganglia, further suggesting a metabolic defect associated with the CAG expansion in the huntingtin protein.

6. Therapeutic strategies

If a defect in energy metabolism underlies the pathogenesis of neurodegenerative diseases then a reasonable therapeutic strategy is to utilize compounds which improve mitochondrial function. Coenzyme Q_{10} or ubiquinone is an essential component of the electron transport chain where it serves as an electron donor and acceptor. It is also a potent antioxidant, particularly in mitochondria. Prior work demonstrated that it protects against glutamate toxicity in cultured cerebellar neurons [115]. We demonstrated that it produces dose-dependent protection against striatal lesions produced by the succinate dehydrogenase inhibitor malonate [111]. More recently we found that it produces marked neuroprotection against 3-nitropropionic acid toxicity, and also improves survival in a transgenic animal model of ALS associated with point mutations and superoxide dismutase (Beal et al., unpublished data). It protects against malonate ATP depletions [111], and it protects against MPTP induced dopamine depletions in older mice [116]. Examination of its effects in HD patients show that it produced a significant 36% decrease in occipital cortex lactate concentrations as assessed by magnetic resonance spectroscopy [98]. Following withdrawal of the coenzyme Q_{10} treatment the lactate levels return to baseline. It produced additive effects with the *N*-methyl-D-aspartate (NMDA) antagonist MK-801 against malonate toxicity in vivo [117]. In view of these neuroprotective effects a clinical trial has been designed to assess the effects of coenzyme Q_{10} with or without an NMDA antagonist in the treatment of Huntington's disease patients.

Another novel therapeutic strategy to ameliorate mitochondrial induced dysfunction is to attempt to buffer intracellular energy stores. The major energy source in the brain is ATP which is tightly coupled to phosphocreatine. A therapeutic strategy may therefore be to administer creatine to attempt to increase phosphocreatine and ATP concentrations within the brain. Creatine kinase catalyzes the reaction of ATP

with phosphocreatine to generate ATP [118]. ATP generated by oxidative phosphorylation is transported through the mitochondrial inner membrane by the adenine nucleotide transporter, where it is transphosphorylated with creatine by the mitochondrial creatine kinase to generate phosphocreatine. Phosphocreatine then leaves the mitochondria and diffuses to the cytoplasm where it serves as both a temporal and spatial energy buffer. Phosphocreatine maintains ATP levels utilized by the sodium potassium ATPase and the calcium ATPase [119]. It serves to maintain membrane potential and to restore ion gradients after neurotransmitter release, consistent with the localization of high amounts of creatine kinase to brain regions rich in synaptic connections [120]. Its importance to brain function is supported by in vivo ^{31}P NMR transfer measurements showing a correlation of creatine kinase flux with brain activity measured by the EEG as well as with brain 2-deoxyglucose uptake [121,122].

Oral administration of creatine stimulates mitochondrial respiration and phosphocreatine synthesis which may help sustain ATP levels under stress conditions [123]. Furthermore phosphocreatine serves as a direct energy source for glutamate uptake into synaptic vesicles [124]. We found that administration of creatine in the diet produced significant protection against both malonate and 3-nitropropionic acid induced neurotoxicity [125]. Creatine increased brain levels of phosphocreatine and ATP and protected against 3-nitropropionic acid induced depletions. Creatine also protected against 3-nitropropionic acid induced increases in striatal lactate concentrations as assessed by proton magnetic resonance spectroscopy. Furthermore creatine administration protected against malonate induced increases in hydroxyl radical generation and increases in 3-nitrotyrosine, and 3-nitropropionic acid induced increases in 3-nitrotyrosine, which may be a downstream consequence of energy impairment. These observations suggest that creatine administration may be a novel therapeutic strategy for the treatment of neurodegenerative diseases.

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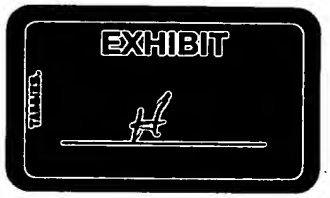

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Brain Metabolism and Brain Disease: Is Metabolic Deficiency the Proximate Cause of Alzheimer Dementia?

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The potential of impairments in oxidative/energy metabolism to cause diseases of the brain had been proposed even before the major pathways of oxidative/energy metabolism were described. Deficiencies associated with disease are known in all the pathways of oxidative/energy metabolism and are associated with some of the most common disorders of the nervous system, including Alzheimer's disease (AD) and Parkinson's disease. A common mechanism in these conditions appears to be a downward mitochondrial spiral, involving abnormalities in energy metabolism, calcium metabolism, and free radicals (reactive oxygen and nitrogen species). In AD, the spiral appears to interact with abnormalities in the metabolism of the Alzheimer amyloid precursor protein (APP) and its Aβ fragment. Several lines of evidence indicate that the mitochondrial spiral may be a proximate cause of the clinical disabilities in AD. Decreases in cerebral metabolic rate (CMR) characteristically occur in AD and in other dementias. Inducing decreases in CMR leads to clinical disabilities characteristically associated with AD and with analogous problems in experimental animals. Treatments directed toward normalizing CMR appear to help at least some patients. Further studies of this possibility and of treatments designed to ameliorate the mitochondrial spiral may prove useful for treating AD and perhaps some other dementing disorders. J. Neurosci. Res. 66:851–856, 2001.

Key words: brain; metabolism; disease; mitochondria; Alzheimer's disease

Recognition of the close dependence of brain function on continuous, efficient utilization of oxygen quickly led investigators to postulate that impairment of cerebral oxidative metabolism might be an important cause of diseases of the brain. This suggestion had been made even before the major pathways of brain metabolism were elucidated. Judah Quastel, one of the founders of modern neurochemistry, stated that hypothesis clearly in the early 1930s:

> The mental symptoms accompanying anoxaemia (as, for instance, that following ascent to high altitudes) are well known. They include loss of judgement and memory, disorientation for time, irritability, and emotional instability. Abnormal mental symptoms accompany carbon monoxide poisoning, and there seems to be little question that anoxaemia of the brain leads to irrational behavior. Anoxaemia may not only be created by lack of oxygen, however, but by conditions set up which render the oxygen unavailable for oxidative purposes. Hence disturbances in the nervous system which result in diminished rates of oxidation will be as productive of mental disorder as lack of oxygen alone (Quastel, 1932).

For the next 40 years, many investigators did not accept that idea. They recognized, correctly, that cutting off the supply of oxygen or glucose killed brain cells, for instance in strokes. They thought, correctly, that measurable falls in adenosing triphosphate (ATP) typically are associated with cell death. However, they assumed, incorrectly, that an impairment of oxidative/energy metabolism severe enough to have any physiological effect would kill cells. That error arose in part because the other functions of mitochondria were not yet appreciated, including their important role in modulating intracellular signal transduction.

Work over the last 30 years has proved Quastel's insight to be prescient. Deficiencies in all the major pathways of oxidative/energy metabolism have been found in patients with brain diseases (Table I). Many of the most common and important diseases of the brain have been found to be associated with deficiencies in these pathways (Table II). The study of oxidative/energy metabolism in diseases of the brain has become a booming area of investigation.

That a variety of clinical syndromes can be associated with disorders of oxidative/energy metabolism is in ac-

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TABLE I. Brain Disorders Associated With Deficiencies in Oxidative/Energy Metabolism*

Pathway	Disorder(s)
Glycogenolosis	Delirium, stupor and coma, MD
Glycolysis	Tremors, hypotonia
PDHC	MD with lactic acidosis, SNE, ataxias, HD, AD
Krebs' tricarboxylic acid cycle	MD with lactic acidosis, ataxia, PD, PSP, AD
Electron transport	Mitochondrial encephalomyopathies, HD, PD, AD
Gluconeogenesis	Lactic acidosis, delirium, stupor and coma
Carnitine acetyltransferase	Ataxia
Glutamate dehydrogenase	Ataxia (OPCA, MSD/PSD)

*MD, mental deficiency; SNE, subacute necrotizing encephalopathy of Leigh; HD, Huntington's disease; AD, Alzheimer's disease; PD, Parkinson's disease; OPCA, olivopontocerebellar atrophy; MSD, multiple system disorder; PSD, Parkinson's spectrum disorder; PSP, progressive supranuclear palsy

TABLE II. Common Brain Diseases With Deficiencies in Oxidative/Energy Metabolism

Disorder	Nature of metabolic deficiency
Delirium	Hypoxia (anoxic, histotoxic, anemic, etc.), hypoglycemia
Wernicke-Korsakoff	Thiamin deficiency: PDHC, KGDHC, transketolase
Pellagra	Niacin deficiency: NAD/NADP-dependent enzymes
Alzheimer's disease	PDHC, KGDHC, COX
Parkinson's disease	Complex I, KGDHC
Huntington's disease	Caudate metabolism, PDHC
Cerebrovascular disease	Decreased O_2, decreased glucose, increased lactate
Schizophrenia?	mtDNA defects?

cordance with current knowledge on genetic brain diseases, i.e., "inborn errors of metabolism" (Scriver et al., 2001). The previously assumed tight association between specific genetic-biochemical defects and specific clinical syndromes has not been borne out by more recent and more extensive studies (Wallace, 1999; Blass and McDowell, 1999; Blass, 2000, 2001; Scriver et al., 2001; Gravel et al., 2001). The earlier view appears to have been due in part to systematic ascertainment errors, which arose because relatively few patients and controls could be studied by the low-throughput methods then available. Screening of larger populations has shown that even classical, hexosaminidase A–deficient Tay-Sachs disease can be associated with at least five distinct syndromes (Gravel et al., 2001). They include schizophrenia and motor neuron disease as well as "classic" Tay-Sachs disease with severe brain damage in infancy and death by 4 years of age. Genetically determined deficiencies of hexosaminidase A occur even in clinically normal people. In general, mutations that lead to functionally milder metabolic deficiencies tend to be associated with milder clinical disorders of later onset, but the relationships are not quantitatively tight (Wallace, 1999; Blass and McDowell, 1999; Blass, 2000, 2001; Scriver et al., 2001; Gravel et al., 2001). As yet, the conventional explanations for the variation of clinical phenotype associated with similar genotypes tend to include relatively vague statements about "genetic background" and "environmental influences" (Wallace, 1999; Blass and McDowell, 1999; Blass, 2000, 2001; Scriver et al., 2001; Gravel et al., 2001). The state of knowledge regarding disorders of oxidative/energy metabolism is similar to that in other metabolic disorders, in which the mechanisms linking different clinical expressions to similar molecular abnormalities are poorly understood (Wallace, 1999; Blass and McDowell, 1999; Blass, 2000, 2001).

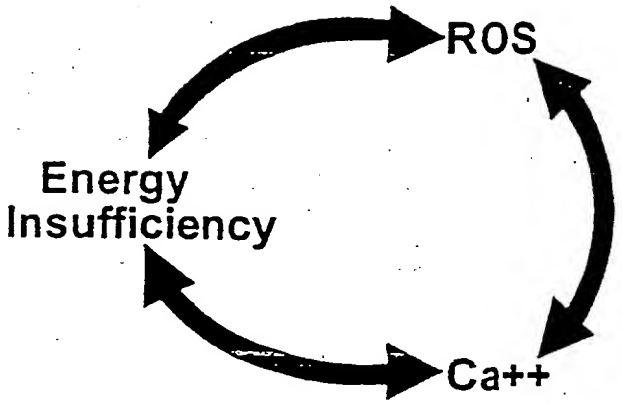

Fig. 1. The mitochondrial spiral. Impairments of energy metabolism, alterations in cellular calcium homeostasis, and excess free radicals (ROS) interact with each other in mitochondria; inducing any one of them leads to abnormalities in the other two. The interaction can set up a deleterious, downward cycle (Blass, 2000)

THE MITOCHONDRIAL SPIRAL

Impairments of oxidative/energy metabolism have a number of consequences in common. One of these is the "mitochondrial spiral" diagrammed in Figure 1 (Blass, 2000). In disorders of oxidative metabolism, abnormalities are typically found in mitochondrial energy metabolism, in calcium metabolism, and in free radicals [reactive oxygen species (ROS) or reactive nitrogen species (RNS)]. These three interact with each other, so that an abnormality in any one of the components of the mitochondrial spiral can be predicted to lead to abnormalities in the other two. A downward spiral is often the result.

Mitochondria are critical subcellular organelles not only for the production of energy but also because they modulate a variety of signaling systems. For instance, mitochondria take up and release calcium; they have a critical role in reducing cytoplasmic calcium in pathological states. Mitochondria are a major source of ROS and RNS, and these radicals have a critical role in modulating cellular functions. For instance, without NO we could not dilate our blood vessels and therefore

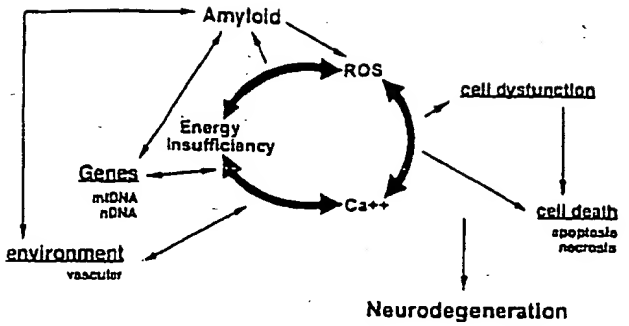

Fig. 2. The mitochondrial spiral in Alzheimer's disease (AD). Either genetic or nongenetic factors can lead to the mitochondrial spiral and to the accumulation of AD amyloid in the brain. Whether the same genes can lead directly to both abnormalities is not known. Vascular disease, which is the most common cause of death in the developed world, is a clinically prominent abnormality, which can lead to increased expression of the AD amyloid precursor protein (APP) and to the mitochondrial spiral. AD amyloid appears to act on cells through free radical mechanisms (Hensley et al., 1996); impairment of oxidative/energy metabolism can lead to increased expression of APP (Kalaria et al., 1993).

could not reproduce our species, at least not by traditional methods. Mitochondrial abnormalities can lead to cell death, both by necrosis and by apoptosis. Mitochondrial proteins such as cytochrome c and apoptosis-inducing factor (AIF) have been shown to play critical roles in apoptotic cell death. Although the mitochondrial spiral itself and a number of its consequences are common to many brain diseases, the precise way in which the spiral interacts with disease-specific abnormalities can obviously vary from disease to disease.

An example is the way in which the mitochondrial spiral may interact with amyloid in Alzheimer's disease (AD). Figure 2 diagrams a possible relationship. Amyloid is thought by many but not all investigators to play a critical role in the pathophysiology of AD. Both genetic and nongenetic factors can lead to increased production of amyloid. Prominent among the nongenetic factors is vascular compromise, as demonstrated in both humans and animal models (Kalaria et al., 1993). Both genetic variations and nongenetic factors can lead to the mitochondrial spiral, and vascular compromise is again prominent among the latter. Whether some of the same genes can lead directly to mitochondrial compromise and directly to amyloid accumulation is uncertain (Sheu et al., 1999). The probable reason why vascular disease is potentially so important in inducing both AD amyloid disease and the mitochondrial spiral is that vascular disease is so common in our species as we age, at least with a modern Western diet. Extensive experimental work by a number of investigators, including Mattson and Markesbery and their coworkers (Hensley et al., 1996), indicates that amyloid (Aβ) can directly induce the mitochondrial spiral. An important way in which it can do so is through free radicals, which can arise when metals are bound to the peptide. Addition of free radical scavengers dramatically reduces the toxicity of amyloid in vitro (Hensley et al., 1996).

Accumulating data from a number of sources indicate that the accumulation of amyloid is not, by itself, adequate to cause dementia (Snowden, 1997; Davis et al., 1999; Wolf et al., 1999). The widely publicized "Nun Study" gave particularly clear results. This is a meticulous clinical and neuropathological study of a community of older religious Sisters in Chicago. Many of the nuns whose brains showed the full *neuropathological* picture of AD, including a high density of neuritic amyloid plaques, were mentally entirely intact during life. They included Sister Mary, who died while mentally sharp on detailed testing when she was 101 years old (Snowden, 1997). (It has been argued that Sister Mary would have been demented if she had lived to age 106 or 110 years. That possibility cannot be checked experimentally. Whether one believes it is a matter of judgment.) The view that amyloid and neuritic plaques in AD are the seminal event in the disease has recently been reviewed (Selkoe, 2001). The neuropathologic data are consistent with the view that neuritic plaques in AD may play a role analogous to that of atherosclerotic plaques in myocardial infarction. Atherosclerotic plaques predispose to the formation of clots, but it is the clotting off of circulation that is the proximate cause of the infarction. We (Blass and McDowell, 1999; Blass, 2000, 2001) and others (Butterfield et al., 1998) have proposed that amyloid plaques may be an important biological risk factor for AD rather than the proximate cause of the clinical signs and symptoms.

The Alzheimer amyloid peptide is, among other things, an antioxidant, because of the properties of its Met_{35} (Butterfield et al., 1998). Expression of the amyloid precursor protein (APP) increases after a variety of insults (Kalaria et al., 1993), suggesting that APP may be an "injury-response protein." These observations suggest that APP expression may increase to protect against oxidative stress. Of course, every reducing agent is potentially an oxidizing agent after it has been converted to the oxidized form. Accumulations of APP or of amyloid in the oxidized form could be a source of free radical damage in AD. That would be consistent with the experimental results and proposal of Butterfield et al. (1998). The situation would then be analogous to the inflammatory cascade. Inability to mount an inflammatory response is life threatening, but excess inflammation has to be treated, for instance, in osteoarthritis.

Another important observation from the Nun Study is that clinically significant dementia is much more likely to develop in people who have both cerebrovascular disease and AD lesions than in people who have AD lesions alone (Hensley et al., 1996). That observation suggests that impaired oxidative/energy metabolism has an important role in the development of the clinical disability. This has contributed to the hypothesis, discussed in more detail below, that the "mitochondrial spiral" is a proximate cause of the clinical dementia in AD.

TABLE III. Koch's Postulates Adapted for Metabolic Disease

1. The metabolic abnormality occurs in the disease state
2. Inducing the metabolic abnormality causes a clinical disorder that mimics the disease state
3. Inducing the metabolic abnormality in experimental animals leads to animal models of the disease
4. Treatment to normalize the metabolic abnormality ameliorates the clinical state

IS THE MITOCHONDRIAL SPIRAL THE PROXIMATE CAUSE OF DEMENTIA IN AD?

Causation is a complicated issue in biology, as has been pointed out by, among others, Aristotle. In the Nineteenth Century, Robert Koch stated four requirements to establish a convincing relationship between an infectious agent and a clinical disease. The following discussion attempts to adapt Koch's Postulates to degenerative disease and specifically to AD (Table III).

Does the Abnormality Accompany the Disease?

Reduction in oxidative/energy metabolism accompanies AD dementia, essentially invariantly. The reductions in cerebral metabolic rate for glucose (CMR_{glu}) and O_2 (CMR_{O2}) and in cerebral blood flow (CBF) are one of the best-documented abnormalities in AD and, indeed, in other dementias as well. They were demonstrated as early as the 1950s by invasive methods and have been reproduced extensively by modern methods, including positron emission tomography (PET), single photon emission computed tomography (SPECT), and functional magnetic resonance imaging (fMRI; Ibáñez et al., 1998). Reduction in CMR characteristically occurs not only in AD but also in most, if not all, of the other nosological entities that cause dementia (Blass and McDowell, 1999; Blass, 2000, 2001). Molecular genetic and family studies have made it possible to identify individuals at high risk for AD before they show clinical signs that can be detected by sensitive neuropsychological tests or evidence of brain atrophy on MRI (Kennedy et al., 1995; Reiman et al., 1996; Johnson et al., 2001). Reductions in CMR occur in these individuals *before* neuropsychological or imaging evidence of AD (Kennedy et al., 1995; Reiman et al., 1996; Johnson et al., 2001). This robustly replicated observation refutes the earlier hypothesis that the reductions in CMR in AD are due to reductions in "brain activity" or to brain atrophy. The temporal relationship—reductions in CMR prior to the development of clinical disabilities—is consistent with the reductions in CMR being a cause of the clinical disabilities. Impairment of oxidative energy metabolism leads to increased expression of the Alzheimer APP (Blass et al., 2000) and to cytoskeletal disorganization, including the appearance of epitopes associated with paired helical filaments/tangles (Blass et al., 1990; Cheng and Mattson, 1992). Impairments of oxidative/energy metabolism are classical causes of premature death of neurons (Blass, 2001). The mechanisms by which abnormalities of oxidative/energy metabolism can contribute to the formation of neuritic plaques and brain atrophy in AD have been reviewed elsewhere (Gibson et al., 1998; Blass et al., 2000; Gibson, 2001).

Defects in AD brain mitochondria have been robustly demonstrated in AD, as discussed elsewhere (Blass and McDowell, 1999; Gibson et al., 2000; Blass, 2000, 2001; Brown et al., 2001). We have proposed that circulatory impairments in the supply of glucose and O_2 to the brain may interact synergistically with inherent impairments in the ability of the brain to oxidize substrate in causing the clinical abnormalities in AD (Blass et al., 2000).

The decrease in CMR is generally proportional to the degree of clinical disability, in studies of groups of patients or in serial studies of single patients (Wolf et al., 1999; Ibáñez et al., 1998). The degree of intrinsic mitochondrial abnormality is also proportional to the degree of clinical disability (Gibson et al., 2000; Brown et al., 2001). This result has been found with two different mitochondrial markers. One is the activity of the α-ketoglutarate dehydrogenase complex (KGDHC) in individuals who possess the *APOE4* susceptibility gene for AD (Gibson et al., 2000). The other is a measure of the amplification of the CO1 gene on mtDNA (Brown et al., 2001).

Does Inducing the Abnormality Lead to the Clinical Signs and Symptoms of the Disease?

Extensive documentation over at least 50 years proves that inducing impairments in brain oxidative/energy metabolism induces abnormalities in memory, judgment, and other higher brain functions that parallel those in AD and other dementias (Gibson et al., 1981; Blass and Gibson, 1999). The effects of reduced oxygen tension in inspired air were studied early and in great detail (Gibson et al., 1981), because of the importance of that knowledge for military aviation in World War II (Fig. 3). Confusion can also be induced by limitations in the supply of sugar (e.g., hypoglycemia) or by vitamin deficiencies that impair the brain's ability to oxidize substrate (e.g., thiamin deficiency), among other causes (Gibson et al., 1981, 1998; Blass and Gibson, 1999; Blass et al., 2000).

Impairing cerebral oxidative/energy metabolism can induce delirium or dementia or both, depending on how severe the metabolic impairment is and for how long it lasts (Gibson et al., 1981; Blass and Gibson, 1999). Delirium is often referred to in neurology as "metabolic encephalopathy" and is characteristically associated with decreased cerebral oxidative/energy metabolism (Gibson et al., 1981; Blass and Gibson, 1999). Delirium and dementia are clinically related conditions. Engel and Romero (1959) conceptualized delirium and dementia as two extremes on a spectrum of "cerebral insufficiency." They put forward this simplifying formulation although they were aware that a variety of etiologies and of nosological entities has been described that can give rise to delirium or to dementia or to both. (Analogously, it is useful to think of febrile seizures as a clinical entity, even though there are many causes of fever.) Engel and Romano (1959) proposed that delirium is functional brain failure and by definition re-

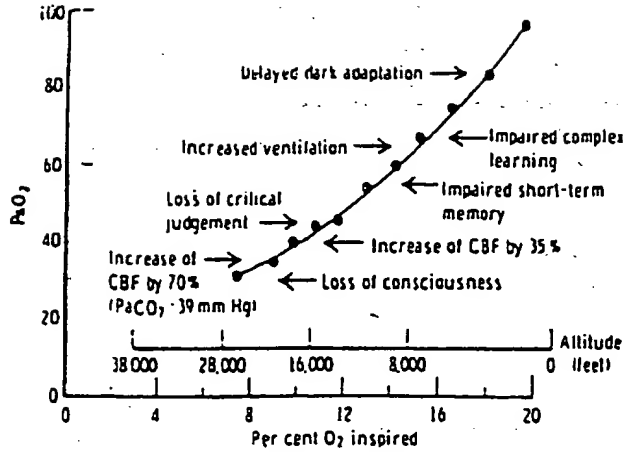

Fig 3. Neurological and psychological effects of hypoxia on humans. This figure, reproduced from the book "The diagnosis of stupor and coma" by Plum and Posner (1980), illustrates the progressive impairment of higher mental functions in humans as the tension of inspired oxygen falls.

versible, whereas dementia is anatomic brain failure and by definition irreversible. In practice, the conditions often overlap. Patients with dementias are particularly susceptible to delirium, even if the dementia is so mild that it is "subclinical" (Gibson et al., 1981; Blass and Gibson, 1999). Chronic delirium can be very difficult to distinguish from dementia. The "reversible dementias" about which much was written some years ago are chronic delirious states.

Impairment of oxidative/energy metabolism can also induce neuropathological changes, which are also known to occur in AD. These include loss of neurons, increased expression of APP, and cytoskeletal abnormalities (Kalaria et al., 1993; Blass et al., 1990, 2000; Blass and McDowell, 1999). The neurons in layers III and V of temporal cortex are selectively vulnerable (i.e., are particularly prone to be lost) in AD, and these neurons are normally enriched in a mitochondrial constituent that is deficient in AD, namely, KGDHC (Ko et al., 2001). This observation has led us to propose that variability in the composition of mitochondria among different kinds of brain cells and among different kinds of neurons may contribute to the selective vulnerability characteristic of AD, and by implication perhaps in other disorders displaying selective vulnerability as well (Ko et al., 2001).

Does Inducing the Abnormality in Experimental Animals Lead to Animal Models of the Disorder?

Common conditions that can induce delirium/dementia in human patients induce analogous conditions in experimental animal models (Gibson et al., 1981; Siegel et al., 1999; Blass and Gibson, 1999). They include, for instance, different forms of hypoxia and of hypoglycemia, ammonia toxicity, deficiencies of vitamins B_1 (thiamine) and B_3 (niacin), and a number of other metabolic insults discussed in detail elsewhere (Siegel et al., 1999). The animals in which these conditions have been experimentally induced have trouble in performing tasks that require learning and skills, including motor skills. These experimental conditions are discussed in standard textbooks (Siegel et al., 1999).

Does Treating the Abnormality Ameliorate the Signs and Symptoms of the Disease?

Two groups have reported that increasing blood glucose improves memory in patients with AD (Craft et al., 1992; Manning et al., 1993). In those studies, blood sugar was clamped at about 220 mg/dl. Craft et al. (1992) have presented evidence that this highly replicable effect is mediated by insulin. That proposal deserves further testing.

At the Burke Medical Research Institute in New York, we have been testing the ability of a patented mixture of glucose and intermediates of the Krebs tricarboxylic acid cycle to ameliorate the clinical illness in ordinary clinical use. In the initial open trial in seven patients, the outcome measure used was the Mini Mental State Examination (MMSE), a standard and robust screening tool for dementia (Anthony et al., 1982). The treated patients improved on average by more than 4 points, and the difference was significant by paired *t*-test. The first attempt at a double-blind study was not truly double blind, because the staff could tell whether the patients were on active preparation or placebo. The results of this effectively open trial agreed qualitatively with those of the intentionally open trial. Patients taking the active, foul-tasting preparation also deteriorated less than those taking the placebo on another standard neuropsychological test used in testing therapies for AD, namely, the ADAS-COG. A true double-blind trial is in progress.

The assumption underlying the Burke trials is that ameliorating the metabolic defect may well help AD patients, *if* the metabolic defect is a proximate cause of their clinical disability. That may be true even if some other abnormality, such as amyloidosis, is a more "fundamental" cause of the disease. An analogy is the use of aspirin or other anticlotting agents to prevent thromboses in patients with atherosclerosis, while at the same time trying to reduce their cholesterol levels.

IMPLICATIONS

A variety of data supports the assumption that the impairment in oxidative/energy metabolism in AD is a proximate cause of the dementia. These data should encourage efforts to ameliorate this abnormality in a clinically useful way.

Several other dementing disorders are also associated with decreases in brain oxidative/energy metabolism (Browne et al., 1997; Mizuno et al., 1998; Albers et al., 2000). Global cognitive impairment with normal cerebral oxidative/energy metabolism appears to be rare. These observations lead to the speculative hypothesis that impairment of brain oxidative/energy metabolism is the proximate cause of many disorders that impair mentation.

Further studies are needed to test this hypothesis, not only in dementias but also in other chronic impairments of mentation, including certain forms of madness. Intensive studies of oxidative/energy metabolism in neurological and psychiatric disorders may yet aid us to provide better help for people suffering from these conditions.

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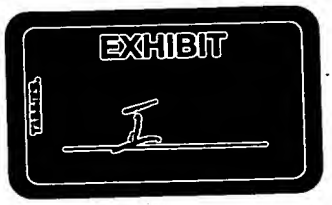

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MINIREVIEW

BIOENERGETIC AND OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASES

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Summary

Aging is a major risk factor for several common neurodegenerative diseases, including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Huntington's disease (HD). Recent studies have implicated mitochondrial dysfunction and oxidative stress in the aging process and also in the pathogenesis of neurodegenerative diseases. In brain and other tissues, aging is associated with progressive impairment of mitochondrial function and increased oxidative damage. In PD, several studies have demonstrated decreased complex I activity, increased oxidative damage, and altered activities of antioxidant defense systems. Some cases of familial ALS are associated with mutations in the gene for Cu, Zn superoxide dismutase (Cu, Zn SOD) and decreased Cu, Zn SOD activity, while in sporadic ALS oxidative damage may be increased. Defects in energy metabolism and increased cortical lactate levels have been detected in HD patients. Studies of AD patients have identified decreased complex IV activity, and some patients with AD and PD have mitochondrial DNA mutations. The age-related onset and progressive course of these neurodegenerative diseases may be due to a cycling process between impaired energy metabolism and oxidative stress.

Key Words: mitochondria, oxidative phosphorylation, electron transport chain, oxidative damage, free radicals, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease

Among the most common neurologic diseases are neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). As the elderly population increases, the prevalence of these age-related diseases is likely to increase. The cause of these diseases is not known, and, with the possible exception of PD, there is no treatment that alters the progression of any of these disorders.

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Of the few risk factors that have been identified for these diseases, increased age is the only one that is common to AD, PD, ALS, and HD. For AD, the incidence and prevalence of the disease increase dramatically with age after age 60; one study showed a 47% prevalence for patients over age 85 (1). Two processes that have been implicated in the aging process are mitochondrial dysfunction and free radical-induced oxidative damage (2, 3, 4). In addition to their possible involvement in aging, mitochondrial dysfunction and oxidative damage may play important roles in the slowly progressive cell death that is characteristic of several different neurodegenerative diseases

Mitochondria and Oxidative Phosphorylation

In different tissues, mitochondria exhibit different sizes, shapes, and densities (4, 5, 6). Each mitochondrion consists of two phospholipid bilayers. the outer membrane and the inner membrane The space between the inner membrane and outer membrane is the intermembrane space The area enclosed by the inner membrane is the matrix, which is the site of the tricarboxylic acid cycle. The inner membrane contains the protein complexes that catalyze oxidative phosphorylation. Five different complexes are involved in oxidative phosphorylation. complex I (NADH: ubiquinone oxidoreductase), complex II (succinate: ubiquinone oxidoreductase), complex III (ubiquinol: cytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase) Complexes I, II, III, and IV constitute the electron transport chain Mitochondrial DNA (mtDNA) is a circular molecule that encodes for two mRNA molecules, 22 tRNA molecules, seven complex I polypeptide subunits, one complex III subunit, three complex IV subunits, and two complex V subunits Nuclear DNA (nDNA) encodes for the remainder of the subunits of the oxidative phosphorylation system

Free Radicals and Oxidative Damage

Free radicals are species that contain one or more unpaired electrons and exist independently (7, 8) Some of the most important free radicals in biological systems are oxygen-centered free radicals, which include inorganic molecules, such as superoxide and hydroxyl radical, and organic molecules, such as alkoxy and peroxy radicals. Through reduction and oxidation reactions, free radicals may damage a variety of macromolecules Free radical-induced oxidative damage includes DNA strand breaks (9), DNA adduct formation (such as 8-hydroxy-2-deoxyguanosine) (10, 11), lipid peroxidation (12), and the generation of protein carbonyl groups (13, 14). Oxidative damage to macromolecules may alter their function and thereby lead to impaired cellular functioning or cell death

Interactions Between Oxidative Phosphorylation and Oxidative Damage

There are significant interactions between oxidative damage and mitochondrial energy metabolism, especially the oxidative phosphorylation system. Oxidative phosphorylation generates most of the free radicals in the cell (15). The components that produce the most free radicals are ubiquinone and cytochrome b_{566} of complex III (16). Free radical generation is increased by inhibition of the electron transport chain (15, 17)

In addition to generating free radicals, the oxidative phosphorylation system itself is vulnerable to damage by free radicals One mechanism by which the oxidative phosphorylation system may be injured by free radicals is through oxidative

damage to the mtDNA. The mtDNA is particularly susceptible to oxidative damage, and all of the polypeptides that are encoded by mtDNA are components of the oxidative phosphorylation system (4, 6). This susceptibility of mtDNA is probably due to its lack of protective histones, limited repair capabilities, and proximity to the electron transport chain (2, 3, 4). The respiratory chain complexes may also be affected directly by reactive oxygen species. In submitochondrial particles, complex I is particularly sensitive to hydroxyl radical and superoxide anion (18). In *in vivo* studies, complex IV is the most vulnerable to peroxidative stress, but complexes I and II are also affected (19, 20). This vulnerability of the electron transport chain complexes may be due to oxidative damage to proteins. Also, since the complexes are membrane-bound and sensitive to the lipid microenvironment (21, 22), oxidative damage to phospholipids of the inner mitochondrial membrane may also be involved.

A cycling process may occur between oxidative damage and oxidative phosphorylation due to the fact that free radicals are injurious to this system that also generates them (Fig. 1). Since oxidative phosphorylation generates free radicals, it is possible that these free radicals damage mtDNA, proteins, and lipids. This damage may then impair oxidative phosphorylation such that greater levels of free

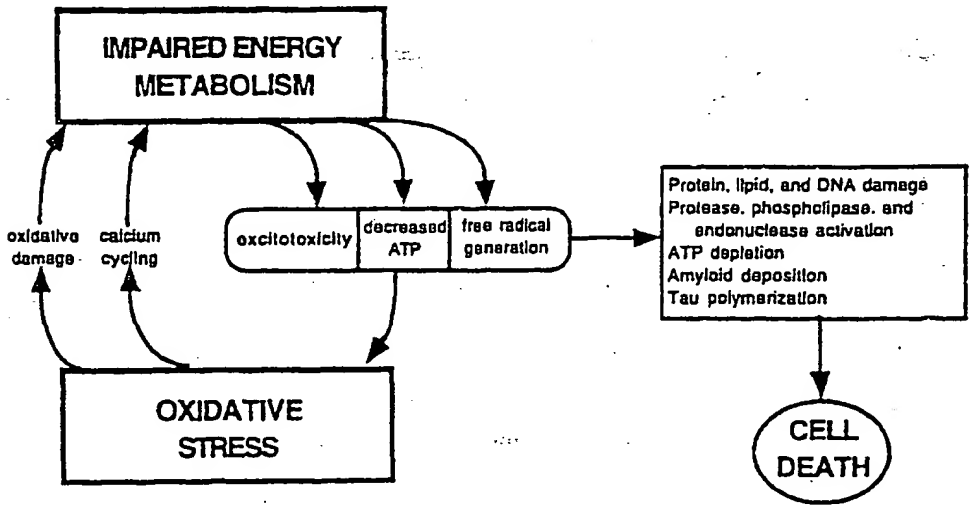

Fig. 1

Possible cycling mechanisms between impaired energy metabolism and oxidative stress. Oxidative stress may produce oxidative damage to macromolecules and "calcium cycling," both of which may impair energy metabolism. Impaired energy metabolism may then result in several processes: excitotoxicity, decreased ATP levels, and increased free radical generation. These three processes may facilitate cycling by further increasing oxidative stress. In addition, these three processes may play an important role in cell death through oxidative damage to macromolecules, excitotoxic mechanisms (protease, phospholipase, and endonuclease activation), ATP depletion, amyloid aggregation, and tau polymerization.

radicals are generated, and these free radicals may result in additional oxidative damage. Another possible cycling mechanism may occur through cytochrome b_{566}; reduced ATP levels result in increased free radical generation by cytochrome b_{566}, such that a slight impairment in oxidative phosphorylation that mildly reduces ATP levels may increase free radical generation, increase oxidative damage to the oxidative phosphorylation system, and then lead to further reductions in ATP levels (23). Also, calcium cycling may play an important role in interactions between energy metabolism and oxidative damage since oxidative stress may impair mitochondrial function due to excessive release and reuptake of mitochondrial calcium (24, 25)

There are several implications of this cycling process between energy metabolism and oxidative damage First, minor defects that mildly impair oxidative phosphorylation or slightly increase oxidative damage may become amplified over time. For example, an impairment in oxidative phosphorylation may initially produce mildly elevated free radical levels These free radicals may then impair oxidative phosphorylation further and lead to even greater levels of free radicals Second, different combinations of environmental and genetic factors that alter mitochondrial function and oxidative damage may produce different rates of amplification and thereby lead to variability in the amounts of oxidative damage observed between individuals Finally, these cycling processes indicate that the pathway that produces cell death may be similar for defects that initially increase oxidative damage and for defects that initially impair mitochondrial function

Energy Metabolism, Oxidative Damage, and Excitotoxicity

Impaired energy metabolism and oxidative damage have important interactions with excitotoxicity. These interactions, which have been reviewed recently (26-29), may produce additional cycling mechanisms that are important for cell death (Fig 1) A full discussion of excitotoxicity is beyond the scope of this review, and, therefore, will be discussed briefly With regard to the N-methyl-D-aspartate (NMDA) receptor, impaired energy metabolism may enhance excitotoxicity by several mechanisms (26), including a sequence of events that involves decreased ATP levels, reduced sodium-potassium ATPase activity, partial cell-membrane depolarization, and relief of the voltage-dependent Mg^{2+} block of NMDA-associated channels Also, activation of NMDA receptors involves the generation of nitric oxide (30), which may impair energy metabolism by two mechanisms: (1) inhibition of mitochondrial enzymes, including complex IV (31, 32) and iron-sulfur cluster-containing enzymes, such as complex I, complex II, and aconitase (31, 33, 34, 35); (2) activation of poly (ADP-ribose) synthetase, which may impair energy metabolism and energy-dependent processes by decreasing intracellular levels of NAD and ATP (36, 37)

Free radicals may play an important role in NMDA and non-NMDA receptor-mediated excitotoxicity NMDA receptor activation has been associated with the generation of superoxide (38) In addition, nitric oxide synthase, which is stimulated by NMDA receptor activation, generates nitric oxide (30) as well as superoxide (39) and hydrogen peroxide (40). These reactive oxygen species may damage and impair the function of a wide range of macromolecules Also, nitric oxide may react with superoxide to generate peroxynitrite, a powerful oxidant (41) Finally, the NMDA receptor contains a redox modulatory site at which oxidized nitric oxide congeners may react with thiol groups to downregulate channel activity (42) Kainate-induced

neurotoxicity is decreased by antioxidants (43, 44) and is associated with generation of free radicals (45) and increased lipid peroxidation (44, 45). Also, the toxicity of quisqualate is decreased by idebenone, an antioxidant (46). Free radicals have been associated with increased release (47, 48) and decreased uptake (49) of excitatory amino acids. We recently demonstrated that free radical spin traps can attenuate NMDA-, kainate-, and AMPA-induced lesions *in vivo* (50). Furthermore, we showed that the spin traps decreased hydroxyl radical generation that was produced by these compounds.

Bioenergetic and Oxidative Stress in Neurodegenerative Diseases

Defects in oxidative phosphorylation and oxidative damage may play an important role in a variety of common neurodegenerative diseases, including PD, AD, ALS, and HD. The progressive course and age-related increase in incidence of these disorders may be due to interactions between oxidative damage and defects in oxidative phosphorylation. These processes may be the primary pathologic process or they may be involved secondarily, either as risk factors that facilitate some other primary pathologic process or as processes that become involved "downstream" to a different primary pathologic event.

Aging

Studies in brain and other tissues have suggested that there may be an age-associated impairment of oxidative phosphorylation. Respiratory activity and complex IV activity are reduced in aged rat brain (51, 52), while activities of complexes I and IV are reduced in aged rat muscle (53). Human studies have demonstrated increased numbers of complex IV-deficient myocytes (54, 55), reduced respiratory activity in intact liver mitochondria (56), and decreased activities of several complexes of the electron transport chain in skeletal muscle (57, 58). In a study of the oxidative phosphorylation system in aged rhesus monkeys, we demonstrated an age-related decline in the activities of complexes I and IV in frontoparietal cortex (59). There were no significant age-associated changes in the activities of complexes II-III and V. A study of ATP production in intact mitochondria isolated from aged squirrel monkeys also indicated that there was an age-associated decline in complex I activity that was most prominent in the caudate (60).

In terms of oxidative damage, there is evidence for age-related increases in several different markers of oxidative damage. The most common free radical-induced DNA adduct, 8-hydroxy-2-deoxyguanosine (8OH-dG), increases with aging in nDNA from several different rat tissues (61). The content of 8OH-dG in mtDNA is 10-fold greater than that of nDNA (62) and increases with aging in human diaphragmatic and cardiac muscle (63, 64). In cortical tissue from human postmortem brain, we found a 10-15-fold increase in the 8OH-dG content of mtDNA relative to that of nDNA (65) The amount of 8OH-dG in nDNA and mtDNA increased with aging, and this aging effect was much more striking in the mtDNA than the nDNA

Aging has also been associated with several different mtDNA deletions The most common aging-associated deletion is a 4977 nucleotide pair (np) deletion (66-68), but deletions of 7436 np (67-70) and 10422 np (68) have also been reported. The 4977 np deletion may be a marker of oxidative damage since the amount of the deletion correlates with the amount of 8OH-dG (64) and it is increased in cardiac tissue of patients dying with ischemic heart disease (67). Our group has

demonstrated age-related increases in the amounts of the 4977 np deletion in human postmortem brain tissue (70). In this study, the age-associated increase was most marked in the putamen; the cerebral cortex exhibited intermediate levels, and the cerebellum exhibited the lowest levels. Similarly, another group found age-dependent increases in the 4977 np deletion in human brain which were most marked in the caudate, putamen, and substantia nigra (71).

Oxidative damage to proteins results in the modification of amino acid residues (72) Carbonyl derivatives are one of the most extensively studied oxidative modifications (72) In postmortem studies of human frontal and occipital cortex, aging has been associated with a two-three fold increase in protein carbonyl content (14, 72). Free radicals may also damage phospholipids with the formation of lipid peroxides. Studies in brain have demonstrated age-related increases in lipid peroxidation (73, 74). These lipid peroxides may alter the function of lipids or may generate additional free radicals that may damage other molecules, such as membrane-associated proteins.

Recent studies in invertebrates have provided additional evidence for free radical involvement in aging In *C elegans*, the age-1 mutation is associated with increased longevity, increased resistance to oxidative stress, and increased activities of catalase and superoxide dismutase (SOD) (75). Transgenic *Drosophila melanogaster* that overexpress catalase and Cu, Zn SOD exhibit increased longevity, decreased protein oxidative damage, and decreased age-associated decline in physical performance (76). In contrast, life-span was not affected or was only minimally increased in transgenic *Drosophila* that overexpress either Cu, Zn SOD or catalase alone (77, 78) Similarly, in mouse epidermal cells transfected with Cu, Zn SOD and catalase, the cells that overproduce only Cu, Zn SOD exhibit increased sensitivity to oxidative stress, while those that overproduce Cu, Zn SOD and catalase are protected from oxidative damage (79). These studies suggest that reactive oxygen species play an important role in the aging process and that significant effects on aging are only observed with concomitant increases in Cu, Zn SOD and catalase activities

The association of aging with the insidious development of impaired mitochondrial function and increased oxidative damage is consistent with a cycling mechanism in which deficits become amplified over time These age-related deficits may be components of normal aging that are an inevitable consequence of the toxicity of aerobic metabolism. In humans and animals, aging is associated with the slowly progressive development of cognitive dysfunction (80-82) and physiologic deficits (80) These age-related processes exhibit variability between individuals (80-82) It is possible that some of these processes are due to combinations of genetic and environmental factors that influence mitochondrial function and oxidative damage. In addition, some of the variability in the rate of decline of these processes (80, 81) may be due to variable combinations of these genetic and environmental factors

Parkinson's Disease

Much of the interest in the association of neurodegeneration with mitochondrial dysfunction and oxidative damage emerged from studies of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism (83) MPTP is converted to 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B (MAO-B) MPP^+ inhibits complex I activity by binding reversibly to a site at or near the

rotenone-binding site. The close relationship between mitochondrial dysfunction and oxidative damage is apparent with MPP^+ toxicity since MPP^+ induces superoxide formation (84), increases lipid peroxidation (84), and, with prolonged exposure, irreversibly inhibits complex I by a mechanism that may be due to oxidative damage to complex I (85).

Substantial evidence has been obtained of a mitochondrial defect in PD. In accordance with the finding that MPP^+ inhibits complex I, the most consistent oxidative phosphorylation defect in PD patients has been reduced complex I activity. In studies of PD brain tissue, complex I activity is decreased in substantia nigra (86-91) and is not altered in other brain regions (89, 90, 92). An immunohistochemical study showed reduced staining for complex I subunits in PD substantia nigra, but preserved staining for subunits of the other electron transport complexes (93). Similarly, decreased complex I activity has been reported in skeletal muscle (94-96), platelets (97-101), and lymphocytes (102). However, in contrast to these findings, some studies in platelets, muscle, and fibroblasts have demonstrated no reduction in complex I activity (90, 92, 99, 103-106) or reduced activity of other complexes (86, 92, 94-96, 99, 100, 102) Alpha-ketoglutarate dehydrogenase complex (KGDHC), a mitochondrial enzyme that is the rate-limiting enzyme in the tricarboxylic acid cycle and provides succinate for complex II, has also been implicated in PD. KGDHC is inhibited by millimolar concentrations of MPP^+ (107), and immunostaining for KGDHC is decreased in PD substantia nigra (108). Some early studies indicated that the 4977 np deletion of the mtDNA was increased in the striatum and cortex of PD patients (109), but this finding has not been replicated in subsequent studies in substantia nigra (110-112), putamen (111), cortex (111), or skeletal muscle (94, 105, 113)

Evidence for increased oxidative stress in PD includes increased lipid peroxidation in the substantia nigra in PD patients (114, 115). The levels of reduced glutathione are decreased in the substantia nigra but not in other brain regions (116-120). Similarly, in a small number of patients with incidental Lewy body disease, which may be an early stage of PD, the only significant biochemical abnormality was a 35% reduction in reduced glutathione levels (121) In some of these glutathione studies, there may have been autolytic loss of glutathione during the postmortem interval (122). Decreased glutathione levels could produce, or result from, increased oxidative stress In PD substantia nigra, the activity of γ-glutamyl transpeptidase, a glutathione degradative enzyme, is increased (123), while the activity of glutathione peroxidase may be decreased (124) or unaltered (123, 125). Decreased catalase activity has been reported in PD substantia nigra (126) Conflicting results have been obtained with SOD activity in the substantia nigra. In one study (127), the activity of Mn SOD was increased and the activity of Cu, Zn SOD was not altered; in another study (125), the activity of Mn SOD was not altered and the activity of Cu, Zn SOD was increased. If the activities of Mn SOD or Cu, Zn SOD are actually increased, then this may represent a compensatory response to increased oxidative stress

It has been hypothesized that increased oxidative stress in the substantia nigra in PD is caused by increased iron levels Iron could increase oxidative stress by the Fenton reaction, in which hydrogen peroxide is converted to hydroxyl radical (8). Total iron levels were elevated in PD substantia nigra (118, 128, 129). The significance of the findings in PD patients is not clear, however, since the levels of ferritin, which influence the amount of iron that is in a free and reactive form, were decreased in one study (130) and increased in another study (118). The increases

in iron may be secondary since iron is also increased in the substantia nigra in MPTP-treated primates (131).

Amyotrophic Lateral Sclerosis

The strongest evidence for a role for oxidative damage in the pathogenesis of a neurodegenerative disease has recently been obtained in autosomal-dominant familial ALS. Familial ALS accounts for approximately 10% of all ALS cases and exhibits pathologic and clinical features that are similar to those of sporadic ALS (132-134). Familial ALS has been associated with missense mutations in the *SOD1* gene, which encodes for Cu, Zn SOD; 11 different missense mutations were initially identified in exons 2 and 4 in 13 different families (135). Subsequently, several additional familial ALS-associated *SOD1* mutations have been identified, including mutations in exons 1 and 5 (136-140).

Familial ALS patients with several different *SOD1* mutations exhibit decreased SOD activity In patients with a codon 4 mutation (ala->val), we found a 40-50% reduction in enzyme activity in postmortem brain tissue, erythrocyte lysates, and lymphoblastoid cells (139, 141). In familial ALS patients with several different *SOD1* mutations, erythrocyte lysate SOD activity was decreased by 50-65% (136) Similarly, in patients with a codon 38 (leu->val) mutation, erythrocyte SOD activity was decreased by 66% in an affected patient and by 60% in seven unaffected carriers of the mutation (142). Erythrocyte SOD activity was decreased by approximately 20% in six patients with a codon 46 mutation (his->arg) (137).

These findings in a variety of tissues indicate that the *SOD1* defect is probably generalized, and yet familial ALS preferentially involves motor neurons. The vulnerability of motor neurons may be due to other factors. Excitatory amino acid toxicity has been implicated in the pathogenesis of ALS on the basis of increased CSF glutamate levels (143), decreased high-affinity glutamate transport in postmortem studies (144), and motor neuron degeneration induced *in vitro* by inhibition of glutamate transport (145). In addition, a recent study reported that riluzole, a glutamate release inhibitor, slowed the progression and improved survival in ALS patients with bulbar-onset disease (146). Activation of excitatory amino acid receptors can increase both superoxide and nitric oxide generation, which may then lead to the formation of peroxynitrite (38, 147)

There are several possible explanations for the finding that to date all familial ALS-associated *SOD1* mutations are associated with decreased enzyme activity. Near-maximal Cu, Zn SOD activity may be necessary for the wild-type phenotype, and, therefore, modest reductions in activity may result in disease. However, most dominantly inherited disorders result in a gain of function (148, 149) Thus, it is possible that there is actually a gain of function and that while the mutations may produce reductions in activity, this reduced activity is not responsible for the disease process. A gain of function is suggested by a recent study in which an ALS-like syndrome occurred in transgenic mice that expressed two wild-type mouse *SOD1* alleles and high copy numbers of mutant human *SOD1* alleles (150). One possible gain of function is that the mutant Cu, Zn SOD reacts more readily with peroxynitrite and thereby generates higher levels of toxic nitronium-like intermediates that lead to increased nitration of tyrosine residues in proteins (151) The function of critical proteins could be altered by these nitrotyrosine residues

Published studies of oxidative damage and mitochondrial function in familial ALS are limited at this time. In Brodmann area 6 (precentral and supplementary cortex) from patients with a codon 4 mutation (ala->val), we found a 20.5% increase in protein carbonyl derivatives; however, this change was not significant (141). Other brain regions that exhibit more marked pathologic changes, such as Brodmann area 4 or spinal cord, may exhibit more oxidative damage Complex I activity was elevated significantly by 55.3% in frontal cortex in familial ALS patients (141). These findings indicate that altered mitochondrial energy metabolism may be associated with the pathologic process.

In sporadic ALS patients, SOD activity levels have been determined in a several different tissues. SOD activity was not significantly altered in postmortem brain tissue (141), erythrocyte lysates (141, 142, 152), or muscle biopsies (152). However, cerebrospinal fluid SOD activity, which is primarily Cu, Zn SOD activity, was decreased in two studies (153, 154)

In a study of oxidative damage in sporadic ALS patients, we found that protein carbonyl groups were increased by 84.8% in frontal cortex (Brodmann area 6) (141) Another group (152) reported no significant change in malondialdehyde, a marker of oxidative damage to lipids, in erythrocytes or muscle biopsies from sporadic ALS patients. The density of glutathione binding sites is increased in the dorsal and ventral horns of sporadic ALS patients (155); this could be due to decreased extracellular concentrations of glutathione or altered modulation of the glutathione binding site

In contrast to our results in familial ALS patients, we did not find any significant alteration in the activities of the electron transport chain complexes in Brodmann area 6 from sporadic ALS patients (141). Ragged red fibers, which are associated with mitochondrial dysfunction in muscle tissue, have been observed in one patient with sporadic ALS (156) Large mitochondria and intramitochondrial inclusions have been observed in hepatocytes from sporadic ALS patients (157-159), and mitochondria with abnormal protrusions have been observed in anterior horn cells of sporadic ALS patients (160) The significance of some of these ultrastructural mitochondrial abnormalities is unclear (161) Support for impaired energy metabolism in sporadic ALS has been obtained in several positron emission tomography (PET) studies that demonstrate widespread reductions in glucose utilization in the cerebral cortex (162-164).

Huntington's Disease

In HD, studies have reported decreased glucose and oxygen utilization in the cortex and basal ganglia of HD patients and some patients at risk for HD (165-171). Recently, with localized proton magnetic resonance spectroscopy, we found increased lactate concentrations in the occipital cortex of HD patients (172). A similar increase in the occipital lactate level has been reported in patients with Kearns-Sayre syndrome, a multisystem neurologic disorder that is associated with deletions of mtDNA (173) In the HD patients, the lactate level correlated with duration of illness (172), and the lactate concentrations decreased when patients were administered coenzyme Q_{10} (174), a compound that may bypass defects in oxidative phosphorylation and act as an antioxidant (174, 175).

Biochemical studies of brain tissue from HD patients have demonstrated multiple defects in the caudate. decreased complex II activity (177); decreased

complex II-III activity and no alteration of complex I or IV activities (178); decreased complex IV activity (179) In platelets, one study reported decreased complex I activity and no change in the activities of complexes II-III and IV (180). Ultrastructural abnormalities in mitochondria have been described in HD cortical tissue (181, 182), and "tweed-ball" mitochondria have been reported in a skin biopsy from a patient with a disease that resembled HD clinically but was not familial (183). Of interest, a point mutation in the ND6 subunit of complex I has recently been associated with basal ganglia degeneration (184)

Studies have not assessed directly whether oxidative damage is altered in HD patients. Lipofuscin accumulation, which may be an indicator of oxidative stress, is increased in frontal cortex (181) In addition, in the basal ganglia, neurons that are vulnerable to degeneration in HD accumulate more lipofuscin than those that are resistant to degeneration (185)

Alzheimer's Disease

In AD patients, PET studies demonstrate reduced glucose metabolism in the temporoparietal region (186-188). These changes occur early in the disease when there is minimal cognitive impairment. Multiple defects in energy metabolism have been identified in AD patients In brain, adenylate energy charge is unchanged but, since oxygen uptake is increased at submaximal metabolic activity, there may be uncoupling of energy metabolism. In these studies, production of $^{14}CO_2$ from [U-^{14}C]-glucose was also increased (189, 190). Skin fibroblasts from AD patients exhibit decreased glucose utilization (191) Several studies of cortical tissue from postmortem brain samples have also demonstrated decreased activities of pyruvate dehydrogenase complex (192-196) and alpha-ketoglutarate-dehydrogenase complex (KGDHC) (195-197) In skin fibroblasts from familial AD patients, KGDHC activity is decreased by 44% and the E2k component of the enzyme complex contains a polypeptide that is absent or present in minimal amounts in controls (198)

In studies of the oxidative phosphorylation system, AD has been associated with reduced complex IV activity We found consistent reductions in complex IV activity in four cortical regions and no consistent alteration in the activities of complexes I, II-III, or V (199) Another study reported decreased complex IV activity in frontal and temporal cortex (200) By complex IV histochemistry, the distribution of activity in the molecular layer of the dentate gyrus was altered and activity was decreased in the dentate gyrus and hippocampal subfields (201) A recent study of purified mitochondria isolated from AD brain tissue demonstrated decreased activity of complex IV and no change in the levels of cytochrome aa_3, the heme-containing component of complex IV (202). These findings suggest that the decreased enzyme activity is due to abnormal catalytic activity rather than decreased enzyme levels. In platelets, Parker et al have demonstrated decreased complex IV activity in mitochondria isolated by two different methods (203, 204). However, with a less pure mitochondrial preparation, there was no significant alteration in complex IV activity (205). If complex IV activity is impaired in AD, then it may result in increased mitochondrial generation of reactive oxygen species. In houseflies, complex IV activity declines with aging and inhibition of complex IV activity is associated with increased hydrogen peroxide production (206). Inhibition of complex IV in submitochondrial particles leads to increased production of superoxide (207).

In an investigation of mtDNA mutations associated with AD and Parkinson's disease (AD-PD), we identified a mutation of a moderately conserved nucleotide (np

4336) in the sequence that encodes for $tRNA^{Gln}$; the mutation was present in 5.2% of patients with AD-PD and in 0.7% of controls (208). Mutations of other mtDNA genes, including a complex I subunit gene, the 12S rRNA gene, and the 16S rRNA gene, were identified in AD-PD patients. These preliminary findings are encouraging but need further confirmation.

There is a limited amount of evidence for increased oxidative damage in AD. We recently found a threefold increase in 8OH-dG levels in mtDNA isolated from cortical tissue of AD patients (209). Also, cortex from AD patients exhibits increased lipid peroxidation (210-213). The activity of glutamine synthetase, an enzyme that may be especially vulnerable to oxidative damage, is reduced in frontal cortex from AD patients (14)

Conflicting results have been obtained in studies of antioxidant defense systems in AD Mn SOD activity was mildly increased in the hippocampus, and Cu, Zn SOD activity was mildly increased in the caudate (214). However, decreased SOD activity has been reported in frontal cortex, hippocampus, and cerebellum (212) Immunoreactivity for Cu, Zn SOD, Mn SOD, and catalase was increased in the region of neurofibrillary tangles and plaques (215). In association cortex and hippocampus from control subjects, high levels of Cu, Zn SOD immunostaining occur in large pyramidal neurons, which are susceptible to degeneration in AD (216). SOD studies in erythrocytes from AD patients have reported no alteration in activity (217, 218) or increased activity (219, 220). In fibroblasts from familial AD patients, SOD activity was significantly increased (221). Erythrocyte glutathione peroxidase activity in sporadic AD has been reported to be normal (217, 218) or increased (222) Fibroblasts from familial AD patients exhibit decreased DNA repair capacity (223), and fibroblasts from familial and sporadic AD patients are more susceptible to oxidative damage (224)

Oxidative stress and mitochondrial dysfunction may interact with other processes that have been implicated in AD pathogenesis. In addition to their possible involvement in excitotoxicity (27), mitochondrial dysfunction and oxidative damage may also facilitate amyloid aggregation and tau polymerization. Amyloidogenic amyloid precursor protein (APP) fragments aggregate in the presence of metal-catalyzed oxidation systems; this effect is inhibited by free radical scavengers (225, 226). In addition, the toxicity of APP fragments in PC12 cells is inhibited by vitamin E and propyl gallate, another antioxidant (227). Amyloid may generate free radicals (228) and inhibit mitochondrial function (229). Oxidation of tau protein appears to facilitate dimerization and polymerization into filaments (230) Also, decreased intracellular ATP concentrations, which could occur with mitochondrial dysfunction, increase the activity of a kinase that may be involved in the hyperphosphorylation of tau (231, 232).

Multisystem Involvement in Neurodegenerative Diseases

If a mechanism involving bioenergetic and oxidative stress is common to the pathogenic process in several different neurodegenerative diseases, then the characteristic clinical and pathologic features of each disease may be due to an interaction with other processes, such as excitotoxicity or amyloid aggregation. Similar underlying mechanisms for these degenerative diseases may result in the coexistence of the features of more than one disease in some patients. In fact, the clinical or pathologic features of more than one neurodegenerative disease have been reported in many patients Pathologic and clinical evidence of PD exist in AD

patients at a frequency greater that that expected by chance (233). Guamanian ALS-parkinsonism-dementia complex patients exhibit various combinations of motor neuron disease, parkinsonism, and dementia (234, 235) Sporadic ALS and Parkinson's disease occur in combination more frequently than expected by chance (233, 236, 237), and, in sporadic ALS cases with no clinical evidence of parkinsonism, pathologic (238-240) and PET studies (241) indicate that there may be nigrostriatal degeneration There may be nigrostriatal degeneration in HD as well (242). There are case reports of sporadic ALS in association with AD (243) as well as other forms of dementia (236, 237, 244-246), including "aphasic dementia" (248), frontal lobe dementia (249), dementia with Kluver-Bucy syndrome (250), and Pick's disease (251). Familial ALS has also been associated with dementia (252-254) HD in association with ALS (255) or AD (256) has been described, but these associations are so rare that they may be fortuitous.

Conclusion

Evidence is accumulating that bioenergetic- or free radical-based mechanisms may play an important role in neurodegenerative diseases While much of the evidence is not definitive at this time, supportive data have been obtained for a wide range of neurodegenerative diseases For some of these diseases, bioenergetic or oxidative stress may be involved in the primary pathologic event, while for other diseases, these processes may facilitate, or result from, a different primary event Further studies are required to clarify whether impaired energy metabolism or oxidative damage are involved primarily or secondarily in the pathogenesis of these disorders For therapeutic considerations, the exact point at which these processes may be involved in cell death may be less important. Whether they are primary or secondary events, mitochondrial dysfunction and oxidative stress may play critical roles in cell death and occur at a time when cells are not irreversibly damaged. If that is the case, then antioxidants and compounds that improve mitochondrial function may slow the degenerative process in several different diseases

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Mitochondria, free radicals, and neurodegeneration

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A central role for defective mitochondrial energy production, and the resulting increased levels of free radicals, in the pathogenesis of various neurodegenerative diseases is gaining increasing acceptance. Defects in energy metabolism may contribute to both excitotoxicity and oxidative damage. Evidence implicating energy defects in neurodegenerative diseases comes from similarities to known mitochondrial disorders, including delayed and variable age of onset, slow progression, and symmetric degeneration of circumscribed groups of neurons.

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Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
HD	Huntington's disease
MND	motor neuron degeneration
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	*N*-methyl-D-aspartate
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
PD	Parkinson's disease
SOD1	Cu/Zn superoxide dismutase 1

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Introduction

The pathogenesis of cell death in neurodegenerative diseases is being intensively investigated. Increasing evidence suggests that defects in mitochondrial energy production may be involved in various neurodegenerative disorders, such as in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). In the case of AD and PD, these defects may be encoded in the mitochondrial genome. A novel technique for investigating energy defects is to create cytoplasmic hybrid, 'cybrid', cell lines, which involves transferring platelet mitochondria from a patient into a mitochondria-deficient cell line. This technique has recently been applied to studies of both AD and PD patients, which I will describe in more detail below. I will also discuss the use of mitochondrial inhibitors in animals as models of both PD and HD. Early pathologic changes in mitochondria appear to also play a role in ALS, as recently demonstrated in a transgenic animal model of the disease.

A consequence of impaired mitochondrial function is the increased generation of free radicals (such as superoxide and hydroxyl radicals), which are normally produced as by-products of oxidative metabolism. An increase in superoxide radical generation can have direct as well as indirect damaging consequences on the metabolism of a cell. Superoxide can combine with nitric oxide (NO) to form peroxynitrite, leading to cytotoxicity; therefore, NO may be a critical component in mediating oxidative damage. In fact, evidence for the involvement of NO in various neurologic diseases is accumulating. Strategies that prevent the generation of NO have shown efficacy in preventing the pathologic features of animal models of both PD and HD. These studies, as well as others implicating oxidative damage in the pathogenesis of neurodegenerative diseases will also be discussed in this review.

Mitochondria, oxidative damage, and neuronal death

Mitochondrial defects that result in a decrease in energy production lead to a number of deleterious consequences. In neurons, this decrease in energy production causes partial neuronal depolarization, which is followed by activation of excitatory amino acid receptors by ambient concentrations of glutamate. Impaired mitochondrial function also impairs intracellular Ca^{2+} buffering. Recent findings indicate that mitochondria are the most important intracellular organelles for buffering large fluxes of Ca^{2+} [1•]. Defects in the mitochondrial electron-transport chain can directly contribute to free-radical production. Mitochondria are known to be the most important physiological source of superoxide radicals in animal cells. Furthermore, an increase in intra-cellular Ca^{2+} leads to increased free-radical generation, and to activation of nitric oxide synthase (NOS), which is dependent on calmodulin.

The interaction of NO with a superoxide radical (O_2^-) leads to the formation of peroxynitrite ($ONOO^-$). This reaction is extremely fast (rate of $6.7 \times 10^9\ M^{-1}\ s^{-1}$), being threefold faster than the rate of dismutation of superoxide by Cu/Zn superoxide dismutase 1 (SOD1). Therefore, the concentration of peroxynitrite depends on the concentrations of superoxide and NO in the cell. Peroxynitrite can exist in an activated transitional form that acts like a hydroxyl radical (OH^-). At physiologic pH, peroxynitrite has a half-life of 0.9 s, allowing it to diffuse over several cell diameters and to cause cell damage by oxidizing lipids, proteins and DNA. The formation of peroxynitrite does not require transition metals.

Recent findings have shown that peroxynitrite ($ONOO^-$) reacts with carbon dioxide (CO_2) to form an unstable nitrosoperoxycarbonate adduct ($O=N\text{-}OOCO^{2-}$) that

appears to rearrange to give a nitrocarbonate anion (O_2N-$OOCO^{2-}$), which may serve as the proximal oxidant in biological systems [2•]. This unstable intermediate can produce one- and two-electron oxidations as well as electrophilic nitration. Nitration reactions that are catalyzed by SOD1 or by reaction with carbon dioxide result in the production of 3-nitrotyrosine, which is used as a specific biochemical marker for peroxynitrite-mediated damage *in vivo* [3]. Nitration of tyrosine residues inhibits the activity of glutamine synthetase [4•], and it disables the ability of tyrosine kinases to phosphorylate the tyrosines [5•], which is a critical event in many cell-signaling pathways and in cell-cycle control.

Evidence for the direct involvement of free radicals in excitotoxic cell death *in vitro* has come from recent studies using the redox-sensitive dyes dihydrorhodamine, dichlorofluorescein and dihydroethidine. These studies have demonstrated a link between glutamate-induced increases in intracellular Ca^{2+} and mitochondrial free-radical production [6•–8•]. A direct role for superoxide and inactivation of aconitase in excitotoxicity has also been demonstrated [9•].

Evidence that NO and peroxynitrite play a critical role in excitotoxic death has been strengthened by observations in cortical cultures from mice deficient in neuronal NOS (nNOS) [10••]. These cultures are resistant to NMDA- and hypoxic-induced neurotoxicity, but are not resistant to toxicity induced by kainate or quisqualate. NOS inhibitors had no effect in these neurons, but did offer protection in wild-type cultures. We have recently shown that 7-nitroindazole, a relatively selective inhibitor of nNOS, protects neurons against NMDA but not against kainate striatal lesions *in vivo* [11••]. The protection was associated with significant reductions in the generation of hydroxyl radicals and 3-nitrotyrosine, consistent with a block of peroxynitrite production. We also found that nNOS-deficient mice are resistant to excitotoxic lesions produced by malonate, and that the resistance is associated with reduced generation of 3-nitrotyrosine [12•]. In contrast, endothelial NOS (eNOS)-deficient mice show increased damage in response to malonate lesions, suggesting that nNOS is critical in mediating toxicity *in vivo*. DNA strand breakage, activation of poly (ADP-ribose) synthetase and cellular energy depletion all appear to play a role in the cytotoxicity of peroxynitrite *in vitro* [13].

Mitochondria and oxidative damage in amyotrophic lateral sclerosis

The observation in 1993 that mutations in SOD1 are associated with some familial forms of ALS suggested that a perturbation in free-radical homeostasis may play a role in its pathogenesis [14]. Substantial evidence indicates that the mutations lead to a gain rather than a loss of function [15]. The most compelling evidence is that overexpression of the human SOD1 mutations associated with ALS in transgenic mice causes motor neuron degeneration (MND), despite normal or increased SOD1 activity [16,17•,18•]. Some of these mice develop cytoskeletal abnormalities similar to those that occur in the human illness [19].

The nature of the gain of function in ALS is also being investigated. The two leading proposals are that the mutations may allow for increased interaction with hydrogen peroxide (H_2O_2) to generate hydroxyl radicals (OH^-), or with peroxynitrite to nitrate proteins. In support of the first hypothesis, a recent *in vitro* study examined two different SOD1 mutations that generated increased hydroxyl radicals, as measured using spin-trapping techniques [20••]. The possibility of increased protein nitration is supported by our findings of significant increases in 3-nitrotyrosine concentrations in the spinal cords of both transgenic ALS mice and human subjects with both sporadic and SOD1-mutation-associated ALS (MF Beal, RJ Ferrante, RT Matthews, NW Kowall, RH Brown, *Soc Neurosci Abstr* 1996, 22:763.9). We have also found increased staining for heme oxygenase, malondialdehyde and 3-nitrotyrosine in the anterior horn motor neurons of ALS patients and transgenic ALS mice. Administering vitamin E, an antioxidant, produces clinical improvements but does not alter survival in transgenic ALS mice [21•].

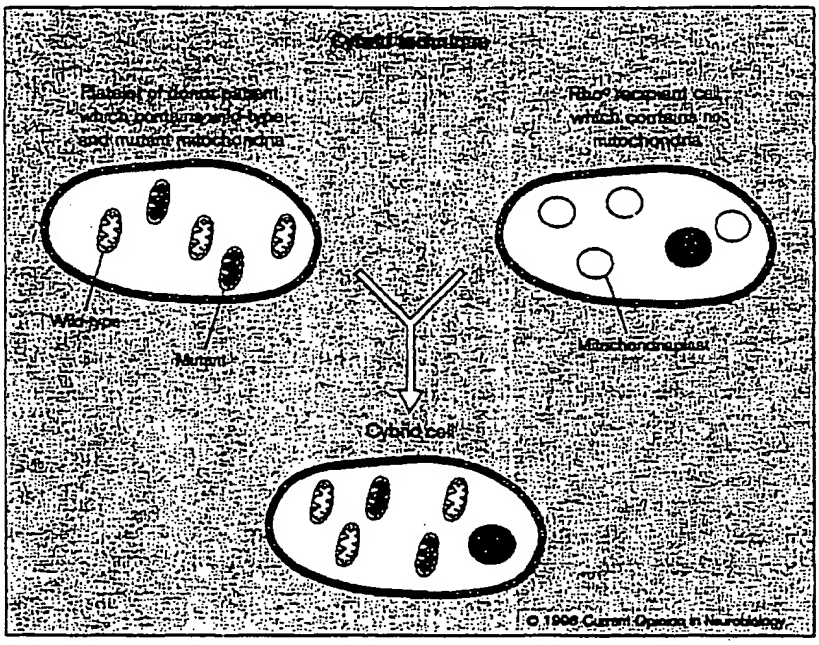
Increased tyrosine nitration might contribute to the pathogenesis of ALS by nitrating tyrosine in neurofilaments, so leading to impaired neurofilament assembly, or by blocking the effects of tyrosine kinases, which mediate the effects of neurotrophic factors. In this regard, it is important to note that defective neurofilament assembly in transgenic mice leads to aggregation of neurofilaments and impaired axonal transport [22••].

A role for mitochondria in ALS is supported by observations in transgenic ALS mice as well as in ALS patients. An early abnormality in the transgenic ALS mice is mitochondrial swelling, leading to splitting of the inner and outer mitochondria membrane and vacuolization [18•]. Presynaptic terminals synapsing on ALS anterior horn cells show aggregated dark mitochondria [23]. Ultrastructural studies of muscle biopsies of ALS patients have shown that motor nerve terminals have significant increases in Ca^{2+}, increased mitochondrial volume and increased numbers of synaptic vesicles relative to controls [24•]. Evidence also supports increased Ca^{2+} levels within mitochondria of the motor nerve terminals of ALS patients.

Mitochondria and oxidative damage in Parkinson's disease

A substantial number of studies have examined PD tissues for mitochondrial defects. The consensus emerging from these studies is that PD is associated with reduced mitochondrial complex I activity in platelets, muscle and the substantia nigra. In support of an energy defect, a recent study used phosphorus magnetic resonance

Figure 1



In the cybrid technique, mitochondria from a donor patient are transferred into a rho^0 recipient (mitochondrial-DNA-free) cell. The resulting cybrid cell contains the patient's mitochondria in a new nuclear background. This technique may be utilized to determine whether observed defects in electron-transport chain enzymes are attributable to mutations encoded by the donor patient's mitochondrial genome

spectroscopy to show a decreased PCr/Pi ratio in resting forearm muscle of PD patients [25]. Furthermore, some PD patients show increased lactate levels in basal ganglia

A novel technique to identify mitochondrial defects that play a role in a disease process is to utilize cytoplasmic hybrid ('cybrid') cell lines, which were pioneered by Giuseppe Attardi (see [26]). This technique has recently been applied to the study of both PD and AD patients. Cybrids are formed by introducing mutant and wild-type mitochondria from a patient's platelets into a human rho^0 cell line that lacks mitochondrial DNA (and is, therefore, devoid of oxidative phosphorylation) (Figure 1) The resulting cybrids enable one to determine whether any defects in oxidative phosphorylation are attributable to alterations in a patient's mitochondrial DNA, as the patient's mitochondria now function in the presence of a different nuclear genome. Using this approach, it was recently demonstrated that mitochondrial complex I deficits in PD patients are expressed in cybrid cell lines, and these deficits are associated with increased free-radical production, leading to apoptotic cell death [27••]. Furthermore, these cell lines showed increased vulnerability to the mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP+), the reactive metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) This is consistent with a potential interaction between mitochondrial defects and environmental toxins in the pathogenesis of PD

Substantial evidence exists for oxidative damage in the substantia nigra in PD patients Immunohistochemical studies have shown increased 4-hydroxynonenal protein adducts in PD substantia nigra neurons [28]. Further evidence implicating oxidative damage in PD pathogenesis comes from studies of MPTP neurotoxicity. MPTP produces a parkinsonian syndrome, both in man and in experimental animals, that closely mimics idiopathic PD. The pathogenesis of the syndrome involves the generation of MPP+, uptake into the dopaminergic nerve terminals, inhibition of mitochondrial complex I and free-radical generation. We recently showed that 7-nitroindazole, a relatively selective inhibitor of nNOS, produces dose-dependent inhibition of MPTP neurotoxicity that is accompanied by reduced striatal concentrations of 3-nitrotyrosine [29••]. These results were confirmed and extended by showing that mice deficient in nNOS are partially resistant to MPTP toxicity [30••]. Furthermore, we found that 7-nitroindazole produces complete protection against dopamine depletion, loss of substantia nigra neurons, and motor and cognitive deficits induced by MPTP in baboons [31••] These findings show that peroxynitrite plays a role in MPTP neurotoxicity and, by implication, in PD.

Mitochondria and oxidative damage in Alzheimer's disease

Substantial evidence suggests that AD is associated with impaired oxidative phosphorylation. We and others have found reduced cytochrome oxidase activity in postmortem cerebral cortex and in platelets [32]. As discussed above, a novel technique for examining mitochondrial defects is to utilize cybrid cell lines. Using this technique, it was recently demonstrated that cytochrome oxidase defects can be transferred to cybrids from AD platelets,

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Previous studies provided evidence of increased oxidative damage in AD postmortem tissue. A recent study utilized novel spin-trapping techniques to show increased oxidative damage to both lipids and proteins [37]. Evidence for oxidative damage at a cellular level is also accumulating. Neurofibrillary-tangle-bearing neurons stain with antibodies to advanced glycation end products, malondialdehyde, carbonylated neurofilaments and heme oxygenase-1 [38–40].

Mitochondria and oxidative damage in Huntington's disease

A role for defects in energy metabolism in HD is strongly supported by both animal studies and lactate imaging using magnetic resonance spectroscopy [41]. A recent study showed that huntingtin, the protein encoded by the HD gene, inhibits the enzyme glyceraldehyde-3-phosphate dehydrogenase as a function of disease-related glutamine repeats [42••]. A report of reduced mitochondrial complex II–III activity in the caudate of HD patients has also appeared [43].

The most compelling evidence implicating oxidative stress in HD comes from animal studies. We have shown that systemic administration of 3-nitropropionic acid, an irreversible inhibitor of succinate dehydrogenase, produces lesions that closely replicate the neuropathologic features of HD. In baboons, a choreiform movement disorder and frontal type cognitive deficits are all characteristic of HD [44••]. The lesions are associated with increases in markers of free-radical damage and are significantly attenuated in mice that overexpress SOD1 [45]. Furthermore, the NOS inhibitor 7-nitroindazole completely blocked the lesions, and this was accompanied by a significant reduction in 3-nitrotyrosine concentrations [11••].

Conclusions

The evidence in favour of mitochondrial defects in neurodegenerative diseases is increasing. In particular, evidence for oxidative damage in the pathogenesis of ALS is accumulating rapidly. The use of cybrid cell technology has made a strong case for mitochondrial defects in the pathogenesis of PD and AD. In HD, it is possible that the huntingtin protein may inhibit glyceraldehyde-3-phosphate-dehydrogenase. Animal models of both PD and HD clearly show an involvement of oxidative damage, and in particular peroxynitrite, in the pathogenesis of neuronal injury. These findings raise the prospect that free-radical scavengers or NOS inhibitors may prove useful in the therapy of neurodegenerative diseases.

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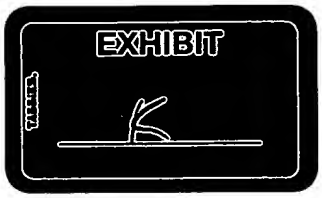

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Oxidative damage and mitochondrial dysfunction in neurodegenerative diseases

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Introduction

During the past few years there have been notable advances towards understanding the molecular defects underlying the pathogenesis of chronic neurodegenerative disorders such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). For example, in 1993, the discovery of the genetic abnormality in HD was reported (an increase in CAG repeats in a gene encoding an unknown protein) [1], while in the same year researchers demonstrated the existence of point mutations in the gene encoding the enzyme Cu/Zn superoxide dismutase (SOD-1) in some familial ALS patients [2]. But despite these findings, the biochemical mechanisms whereby molecular defects can lead to the slowly progressive neuronal degeneration with age-dependent onset, characteristic of these neurodegenerative disorders, still remain an enigma. However, evidence has been mounting in support of the proposition that defects in mitochondrial energy metabolism play an intrinsic role. Further, strong evidence is emerging indicating that this mitochondrial energy dysfunction may result from oxidative damage to mitochondrial DNA and other neuronal macromolecules, and excitotoxic mechanisms have also been linked with mitochondrial dysfunction. Consequently, it has been proposed that a progressive impairment of mitochondrial oxidative phosphorylation may secondarily result in slow excitotoxic neuronal death, by making neurons more vulnerable to endogenous levels of glutamate [3,4]. In this paper, we will review evidence supporting roles for oxidative damage and mitochondrial energy dysfunction in the pathogenesis of neurodegenerative diseases, as well as theories concerning the mechanisms involved, and the initial results of potential therapeutic strategies to ameliorate the effects of mitochondrial dysfunction in these disorders.

Abbreviations used: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; 3-AP, 3-acetylpyridine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; HD, Huntington's disease; MPP^+, 1-methyl-4-phenylpyridinium; mtDNA, mitochondrial DNA; NMDA, *N*-methyl-D-aspartate; 3-NP, 3-nitropropionic acid; OH^8dG, 8-hydroxy-deoxyguanosine; PD, Parkinson's disease; p.e.t., positron emission tomography; SOD, superoxide dismutase; S-PBN, *N-tert*-butyl-α-(2-sulphophenyl)-nitrone.

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Mitochondrial metabolism defects in neurodegenerative disorders

Findings of region-specific decreases in glucose utilization in a number of neurodegenerative disorders in humans led to the suggestion that compromised energy metabolism in neurons may contribute to the pathogenesis of degenerative processes [4]. In AD, for example, positron emission tomography (p.e.t.) studies have shown reduced glucose utilization in the temporo-parietal cortex of AD patients [5,6]. P.e.t. studies have also revealed hypometabolism in caudate and putamen of HD patients, the principle loci of neuronal cell loss in this disease [7–9]. This hypometabolism has been shown to precede the bulk of tissue loss in HD patients, and is also evident in some patients at risk of HD. In addition, we have recently used proton n.m.r. imaging to measure cerebral lactate concentrations *in vivo*, and have found marked increases in lactate levels in the basal ganglia of HD patients (increased 8-fold relative to controls), and in HD cerebral cortex (increased 3-fold), indicative of a defect in energy metabolism [10]. Further, this study also demonstrated a significant correlation between duration of the disease and the extent of lactate increase in the occipital cortex of HD patients. Because oxidative phosphorylation in the mitochondria is the major source of energy generation within cells, these observations stimulated the suggestion that mitochondrial energy metabolism is impaired in neurodegenerative disorders, a theory which has gained substantial support from numerous subsequent investigations into the activities of mitochondrial enzyme systems, discussed below.

Mitochondria comprise an outer membrane, which is freely permeable to macromolecules, surrounding a relatively impermeable inner mitochondrial membrane, which in turn encloses a matrix compartment. Under aerobic conditions, pyruvate (generated by glycolysis of glucose in the cytosol) enters the Krebs cycle located in the mitochondrial matrix. The Krebs cycle generates NADH and $FADH_2$, which then act as electron donors for the electron-transport chain situated on the adjacent

inner mitochondrial membrane. The electron-transport chain comprises an integrated system of five protein complexes which catalyse the phosphorylation of ADP to ATP, with concomitant generation of potential energy. Complexes I (NADH ubiquinone oxidoreductase), II (succinate ubiquinol oxidoreductase), III (ubiquinol cytochrome *c* oxidoreductase) and IV (cytochrome *c* oxidase) are electron carriers which act in series to oxidize NADH and $FADH_2$, ultimately transferring electrons to oxygen. The transfer of electrons also involves pumping of protons across the inner mitochondrial membrane, resulting in the generation of an electrochemical gradient. Complex V (ATP synthase) utilizes this potential energy as an energy source for the high-energy bonds in ATP. Thus, optimal mitochondrial function is dependent on the efficiency of electron movement along the electron-transport chain, and its coupling to oxidative phosphorylation, and therefore any processes which disrupt electron transfer may potentially decrease energy metabolism by mitochondria.

Consistent with this theory, numerous biochemical studies in human post-mortem tissue have identified abnormalities in mitochondrial enzyme activities in aging, and in several neurodegenerative diseases. These have previously been extensively reviewed [4,11]. In brief, reduced complex I activity has consistently been reported in the substantia nigra of PD patients [12,13], supported by immunohistochemical evidence of a down-regulation of complex I subunits in this region, whereas other complex subunits are unaltered [14]. In HD, multiple enzyme defects have been found in the basal ganglia, although at present there is some discrepancy in the changes reported by different groups, with reports of decreased complex II activity [15], decreased complex IV activity [16], and one report of decreased complex II–III activity, but no alteration in complexes I or IV [17], in the caudate of HD patients. Studies in this and other laboratories have also implicated reduced complex IV activity in AD, with the finding of consistent reductions in complex IV activity in four cortical regions [18–20].

In order to elucidate further the role of mitochondrial metabolism dysfunction in neurodegenerative processes, the effects of mitochondrial toxins have been investigated *in vivo* in animal models. These studies have shown that certain mitochondrial inhibitors produce discrete central nervous system lesions which closely mimic the neurochemical and neuropathologic features of particular neurodegenerative disorders. For example, chronic systemic administration of 3-nitropropionic acid (3-NP), a succinate dehydrogenase inhibitor which inhibits both the Krebs cycle and complex II of the electron-transport chain, has been found to produce profound motor disturbances and basal ganglia lesions characteristic of HD in both rodents [21] and in man [22]. Chronic low-grade administration of 3-NP to rats resulted in a depletion of striatal spiny neurons, yet preservation of striatal afferents and aspiny neurons, similar to observations in HD. Furthermore, Golgi studies of spiny neurons showed proliferative changes in their dendrites which are characteristic of HD [23]. We have also found that the extent of 3-NP-induced lesions varies age-dependently [24]. The niacinamide antagonist 3-acetylpyridine (3-AP) is also a potent neurotoxin following systemic administration, producing chronic motor and behavioural abnormalities and selective neuronal degeneration in rodents. In contrast to 3-NP, 3-AP induces neuronal death preferentially in the inferior olive and substantia nigra, and consequently 3-AP lesions have been proposed as a possible animal model of olivopontocerebellar atrophy with associated Parkinsonism [25]. We have also recently shown that 3-AP induced striatal lesions are accompanied by ATP depletions [26].

Putative mechanisms of mitochondrial damage: oxidants and excitotoxicity

Disruption of the electron-transport chain might occur as a primary or secondary consequence of a basic gene defect. One potential mechanism whereby mitochondrial dysfunction could occur is increased generation of free radicals and oxidants. Free radicals including superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($HO^{\cdot}$) are constantly produced as by-products of aerobic metabolism, but under circumstances of electron-transport chain inhibition or molecular defects, levels of free-radical production increase [27,28]. These agents can induce oxidative damage to cell macromolecules including DNA, proteins and lipids by a number of different mechanisms, including induction of DNA strand breaks or formation of DNA adducts such as 8-hydroxy-deoxyguanosine (OH^8dG) [29], forming protein carbonyl derivatives [30], or by lipid peroxidation [31]. One proposal as to how free-radical damage can account for the slow, progressive nature of the neuronal injury occurring in chronic neurodegenerative disorders involves cycling of free radicals and mitochondrial dysfunction. Minor defects in oxidative phosphorylation might enhance the production of free radicals in the mitochondria,

resulting in turn in amplification of DNA mutations and damage to oxidative phosphorylation enzymes, with subsequent up-regulation of free-radical production. This hypothesis of free-radical damage to mitochondrial components is further supported by observations that a number of the subunits of the enzyme complexes comprising the transport chain are encoded by mitochondrial DNA (mtDNA). Seven of the 26 complex I subunits are encoded by mtDNA, as well as one of the 11 complex II subunits, three out of 13 complex IV, and two of the five complex V subunits. The rest are encoded by nDNA. However, the subunits encoded by mtDNA are thought to be particularly susceptible to oxidative damage due to the localization of mtDNA in the mitochondrial matrix, the lack of protective histones, and limited repair mechanisms [32].

Consistent with this view, there is much evidence of increased oxidative stress in neurodegenerative disorders. Increased lipid peroxidation and reduced levels of glutathione have been reported in the substantia nigra of PD patients [33], as well as decreased activities of glutathione peroxidase and catalase which might increase the vulnerability of the substantia nigra to oxidative damage [34]. In studies utilizing the measurement of OH^8dG levels as a marker of oxidative damage to mtDNA, we have found a 3-fold increase in OH^8dG in mtDNA isolated from cerebral cortex of AD patients [35]. However, the strongest evidence to emerge for the involvement of oxidative damage in the aetiology of a neurodegenerative disease comes from the the demonstration of decreased superoxide dismutase (SOD) activity in familial ALS patients, which is consistent with the recently reported finding of mutations in the SOD-1 gene [36,37]. We found that SOD activity was reduced by 40–50% in postmortem brain (and in other tissues), and that the extent of enzyme impairment depended on the locus of the point mutation. Furthermore, the incidence of protein carbonyl groups were found to be increased in the cerebral cortex of patients with sporadic ALS [36].

It is also of interest that markers of oxidative damage increase in incidence with age. We have found a striking age-dependent increase in oxidative damage to mtDNA in cerebral cortex in man, with patients over 70 years of age exhibiting 15-fold increases in levels of OH^8dG in mtDNA, compared with nDNA [38]. Carney and colleagues [39] have also shown that levels of brain protein oxidation increase with age. These observations suggest that a gradual accumulation of oxidative damage with age might account for the slow, insidious onset and progression of neuronal injury in neurodegenerative diseases.

Another mechanism which might putatively account for the slow degeneration seen in neurodegenerative disorders proposes that impaired energy metabolism may enhance excitotoxic processes mediated by the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors [4,40]. Impairment of mitochondrial energy metabolism will result in decreased ATP production, leading to several alterations in cellular physiology, including reduced Na^+/K^+-ATPase activity [41,42]. If the disturbance of ionic pumping at the cell membrane is sufficiently extensive, partial cell depolarization may occur with subsequent alleviation of the voltage-dependent Mg^{2+} blockade of NMDA-receptor-associated channels. This could facilitate activation of NMDA receptors by endogenous levels of glutamate, ultimately leading to increased Ca^{2+} influx into neurons, which has been implicated as a trigger for further free-radical production and damage to cellular elements. In support of this process, a number of studies have shown that ambient, and usually non-toxic levels of excitatory amino acids become toxic in the presence of inhibitors of oxidative phosphorylation [40,43], inhibitors of the Na^+/K^+-pump [40], or partial cell-membrane depolarization by K^+ [43]. Further, a number of recent *in vivo* studies in rats have shown that cerebral lesions induced by mitochondrial toxins such as amino-oxyacetic acid, 1-methyl-4-phenylpyridinium (MPP^+, putatively resembling PD lesions), malonate and 3-AP can be blocked by excitatory amino acid antagonists [26,44]. In addition, the involvement of excitotoxic processes in 3-NP toxicity is supported by evidence that 3-NP-induced lesions show histologic characteristics of excitotoxicity (such as neuronal and dendritic swelling [21]), and a report that 3-NP toxicity can be inhibited by NMDA-receptor antagonists [22]. Furthermore, we have shown that 3-NP lesions are attenuated by prior decortication *in vivo* [45].

Antioxidants and other therapeutic strategies

In light of the evidence of impaired energy metabolism and oxidative damage in neurodegenerative disorders, we have attempted to investigate in animal models the efficacy of agents which putatively ameliorate these events, as potential therapeutic strategies. One approach is to determine whether agents which enhance activity of the electron-transport-chain enzymes can inhibit lesions produced by

mitochondrial toxins. Coenzyme Q_{10} potentiates complex I and II activities in the electron-transport chain, hence increasing ATP production [46], and has been shown to protect against glutamate-induced neurotoxicity in cerebellar cultures [47] In addition, studies in humans have shown that administration of coenzyme Q_{10} to HD patients significantly ameliorates the increased cerebral lactate levels seen in HD [48]. Therefore, we investigated the neuroprotective properties of coenzyme Q_{10} against striatal lesions induced in rats by the complex-II inhibitor malonate. The effects of putatively increasing the availability of the electron-transport-chain substrate NADH were also investigated by assessing malonate lesion volume following pretreatment of rats with the NADH precursor nicotinamide. Chronic treatment with coenzyme Q_{10} or nicotinamide produced dose-dependent protection against malonate lesions, whereas a combination of coenzyme Q_{10} and nicotinamide produced additive neuroprotective effects, enhancing the degree of protection induced by coenzyme Q_{10} alone by more than 100% [49]. Coenzyme Q_{10} pretreatment also prevented the ATP depletion seen in control animals following malonate lesions, whereas nicotinamide in combination with coenzyme Q_{10} actually increased ATP levels in both the lesioned and the contralateral unlesioned striata [49]

An alternative approach to try to prevent oxidative damage is to ameliorate the effects of free radicals Free-radical formation can be reduced by administration of free-radical scavengers such as the spin-trapping agent *N-tert*-butyl-α-phenylnitrone [39] Therefore, we recently examined the effects of free-radical scavengers on glutamate-mediated neurotoxicity and mitochondrial toxin-induced cerebral lesions *in vivo*. Results demonstrated that systemic administration of the spin traps *N-tert*-butyl-α-(2-sulphophenyl)-nitrone (S-PBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) significantly attenuated lesions produced in rats by intrastriatal injection of the excitotoxins NMDA, kainic acid and α-amino-3-hydroxy-5-methyl-4-isoxazole proprionate, and the mitochondrial toxins MPP^+, malonate and 3-AP [50] In addition, in order to further elucidate the nature of the neuroprotective action of S-PBN, we measured its effect on hydroxyl ($HO^{\cdot}$) radical production following mitochondrial toxin lesions, by determining the extent of salicylate hydroxylation to 2,3- and 2,5-dihydroxybenzoic acid (DHBA) S-PBN treatment attenuated the increase in 2,3-DHBA levels seen following intrastriatal malonate injections, consistent with a free-radical effect [50]

Conclusions

In conclusion, the strongest evidence available so far in support of a common neurodegenerative mechanism in disorders such as AD, HD, PD and ALS, is their age-dependent onset and evidence of oxidative stress. Consequently, agents that stimulate mitochondrial energy metabolism, antioxidants, and free-radical scavengers potentially offer not only tantalizing therapeutic opportunities for the treatment of chronic neurodegenerative disorders, but also provide novel strategies by which to gain further insight into mechanisms of neuronal injury in disease

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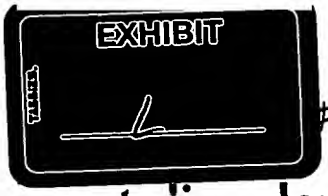

Mitochondrial dysfunction in movement disorders

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A major theory regarding the mechanism of neuronal degeneration in several movement disorders is that mitochondrial defects may play a role. Biochemical studies in Parkinson's disease, Huntington's disease, multiple system atrophy, and idiopathic dystonia have shown defects in enzymes of oxidative phosphorylation in postmortem brain tissue, platelets, muscle, or lymphocytes. The basal ganglia and substantia nigra are also particularly susceptible to the accumulation of age-dependent mitochondrial DNA deletions, which may contribute to the delayed onset of movement disorders. The 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine model of Parkinson's disease involves conversion to 1-methyl-4-phenylpyridinium, which then inhibits complex I of the electron transport chain. Our studies show that the complex II inhibitor 3-nitropropionic acid can closely replicate the neurochemical, histologic, and clinical features of Huntington's disease. The mechanism of neuronal death in both these models may be slow excitotoxicity. Both direct biochemical studies and animal models of movement disorders therefore suggest that mitochondrial dysfunction may play a direct role in their pathogenesis.

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The concept of diseases involving defects in oxidative phosphorylation was first formulated in 1962 when a young woman was found to have a rare hypermetabolic disorder, which is now known as Luft's disease [1] Subsequent studies focused on neuromuscular disorders, which were associated with ragged-red fibers on muscle biopsy results. In recent years, the complete mitochondrial DNA sequence was determined. Subsequently, large mitochondrial DNA deletions were found in patients with Kearns-Sayre syndrome, and point mutations were found in transfer RNA genes of mitochondrial DNA in myoclonic epilepsy with ragged-red fibers and mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes [2] These rare mitochondrial diseases share clinical (ataxia) and pathologic (lesions in the cerebellum and basal ganglia) features with more common movement disorders Subsequent studies have indicated that mitochondrial function in Parkinson's disease (PD), Huntington's disease (HD), and idiopathic dystonia may be reduced, but these findings were questioned by other groups. Our review addresses the possibility that mitochondrial dysfunction is a common feature in the pathogenesis of movement disorders.

Parkinson's disease

A role for dysfunction of the mitochondrial electron transport chain in idiopathic PD was suggested by the analysis of the mechanism of action of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) MPTP produces parkinsonism in humans and primates The toxicity of MPTP is due to its metabolism by monoamine oxidase B in glia to produce 1-methyl-4-phenylpyridinium (MPP^+), which accumulates in dopamine neurons via the dopamine reuptake system and is then concentrated in mitochondria. Within mitochondria, MPP^+ acts to inhibit NADH-coenzyme Q reductase (complex I) of the electron transport chain *in vitro* and *in vivo*, thereby leading to a fall in ATP production and thus initiating cell death [3••]

The role of mitochondrial dysfunction in PD has received considerable attention Studies of the electron transport enzymes have been carried out in platelets, lymphocytes, muscle biopsies, and postmortem brain tissue. Of the five complexes of the respiratory chain, a decrease in the activity of complex I has been the most consistent finding in brain, platelets, muscle, and lymphocytes (Table 1). The most convincing evidence of complex I deficiency in PD comes from studies of brain tissue [4–6] Complex I activity was reported to be decreased in substantia nigra tissue homogenates but not in the caudate, cerebral cortex, cerebellum, or either segment of the globus pallidus. There were no changes in complex II-III or complex IV activities In patients with multiple system atrophy (MSA) who share clinical features and degeneration of the substantia nigra with patients with PD, complex I activity was normal [5] Although these studies were only conducted by a single group, great care was taken in considering age, medication, agonal period, brain pathology, and

Abbreviations

HD—Huntington's disease; MPP^+—1-methyl-4 phenylpyridinium; MPTP—1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSA—multiple system atrophy; PD—Parkinson's disease.

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time between death, autopsy, and freezing These variables may influence secondary changes in mitochondrial enzyme activity. The specificity of this enzyme defect and its selectivity for the substantia nigra support a pathogenic role.

Consistent with these findings was that immunohistochemical studies using antisera against complexes of the electron transport chain showed that melanin-containing nigral neurons in PD brains had reduced staining with a complex I antibody as compared with control subjects [7] Therefore, the complex I abnormality was localized to the melanin-containing nigral neurons Unfortunately, no information was given by the authors about the staining pattern of neurons with Lewy bodies. Recently, it was shown histochemically that in addition to a decrease of complex I activity there may be a loss of the α-ketoglutarate dehydrogenase complex in the substantia nigra [8•] The α-ketoglutarate dehydrogenase complex is an important enzyme complex of the tricarboxylic acid cycle It catalyzes the oxidation of α-ketoglutarate, providing succinate through succinyl semialdehyde. Succinate serves as a substrate for complex II. Therefore, if α-ketoglutarate dehydrogenase is inhibited, electron transport through complex II will also be disturbed. In this situation, neurons will be unable to use complex II to compensate for a complex I deficiency A loss of complex I and α-ketoglutarate dehydrogenase are therefore likely to impair ATP synthesis

Complex I is comprised of approximately 41 different polypeptide subunits Seven of the subunits are encoded by mitochondrial DNA, whereas the rest are

Table 1. Activities of the electron transport chain in different tissues of patients with Parkinson's disease

Study	Tissues examined	Patients/control subjects examined, *n/n*	Defect of ETC*	Age-matched	Method
	Brain				
Schapira *et al* [4]	SN	9/9	Complex I (30%)	Yes	Activities of ETC enzymes
Mizuno *et al* [10]	Caudate and putamen	5/5	Complex I	No	Immunoblot analysis
Schapira *et al.* [5]	SN, caudate, pallidum cortex and cerebellum	7/6	Complex I (42%) in SN only	Yes	Activities of ETC enzymes
Hattori *et al.* [7]	SN	8/7	Complex I (staining reduced in 13% to 74% of neurons)	No	Immunohistochemistry
Mann *et al* [6]	SN, tegmentum, and cerebellum	17/22	Complex I (36%) in SN only	Yes	Activities of ETC enzymes
	Muscle				
Bindoff *et al.* [16]	Mitochondrial fraction	5/4	Complex I (40%), II (49%), IV (40%)	Yes	Activities of ETC enzymes
Shoffner *et al* [17]	Mitochondrial fraction	6/16	Complex I (2P), I, II, III (2P), II, III, IV (1P); Normal (1P)	?	Activities of ETC enzymes
Nakagawa-Hattori *et al* [18]	Mitochondrial fraction	4/6	Complex I (49%)	Yes	Activities of ETC enzymes
Mann *et al* [6]	Mitochondrial fraction	9/6	Normal	Yes	Activities of ETC enzymes
Anderson *et al.* [21•]	Mitochondrial fraction	7/6	Normal	Yes	Oxygen consumption and activities of ETC enzymes
Di Donato *et al* [20•]	Mitochondrial fraction	6/6	Normal	Yes	Activities of ETC enzymes
Di Donato *et al* [20•]	Crude homogenates	16/8	Normal	Yes	Activities of ETC enzymes
Cardellach *et al.* [19•]	Mitochondrial fraction	8/10	Complex I (35%), IV (68%)	Yes	Activities of ETC enzymes
Di Monte *et al* [22•]	Intact mitochondria	11/11	Normal	Yes	ATP measurement
	Platelets				
Parker *et al* [11]	Mitochondrial fraction	10/8	Complex I (54%)	Yes	Activities of ETC enzymes
Mann *et al.* [6]	Crude homogenates	14/15	Normal	Yes	Activities of ETC enzymes
Yoshino *et al.* [13]	Crude homogenates	20/17	Complex I (26%); II (19 6%)	Yes	Biochemical assays for activities of ETC enzymes
Bravi *et al.* [12]	Mitochondrial fraction	17/13	Normal	Yes	Oxygen consumption and activities of ETC enzymes
Krige *et al.* [14]	Mitochondrial fraction	15/25	Complex I (16%)	Yes	Activities of ETC enzymes
Benecke *et al* [15•]	Mitochondrial fraction	17/44	Complex I (52%); IV (30%)	Yes	Activities of ETC enzymes
	Lymphocytes				
Yoshino *et al* [13]	Crude homogenates	20/17	Complex II (13%)	Yes	Biochemical assays for activities of ETC enzymes
Barroso *et al.* [61]	Crude homogenates	16/15	Complex I (14%), IV (45%)	Yes	Biochemical assays for activities of ETC enzymes

*The decrease of the enzyme activity of particular enzymes is given in parentheses
ETC—electron transport chain; P—patients; SN—substantia nigra

nuclear encoded. Attempts to determine whether one of these subunits is defective in PD gave varying results The Western blot pattern was normal in one study [9], but in another it suggested a selective decrease of lower molecular weight subunits [10]

Parker *et al* [11] initially reported a 54% decrease in complex I activity in mitochondrial fractions of platelets in 10 patients with PD compared with that of eight control subjects Subsequent work using platelet homogenates [6] or platelet mitochondria–enriched fractions [12] did not confirm a decrease in complex I activity Other studies, however, have confirmed small, significant decreases in platelet complex I activity using crude homogenates [13] or enriched platelet preparations [14]. Recently, Benecke *et al* [15•] reported decreases of 52% and 30% of the activities of complexes I and IV, respectively, in mitochondrial fractions of platelets of patients with PD but reported normal activities in patients suffering from MSA. Repeated measurements in five patients with PD, with an initial assessment of enzyme activities at an early stage of the illness and a second measurement 1 year later, demonstrated a significant decrease in the activities of complexes I and IV This finding suggests that, at the beginning of the disease or in a presymptomatic stage, the decrease of complex I activity is small but increases during disease progression. However, this finding is contrasted by lack of correlation between disease duration and enzyme activities in patients with PD who are in a more advanced stage of their disease [6,14,15•].

Bindoff *et al* [16] initially found decreased levels of complexes I, II, and IV in muscle A study of six patients with PD showed normal respiratory chain function in one patient, pure complex II defects in two patients, complex I-III defects in another two patients, and low activity of complexes II-IV in one patient [17]. A study of muscle at autopsy showed reduced complex I activity, whereas the other electron transport enzymes showed normal activities [18]. Another recent study showed reduced activities of both complex I and IV, yet showed normal complex II and III activities [19•]. However, other groups reported normal activities in the electron transport enzymes in muscle homogenates or mitochondrial fractions [6,20•,21•] or normal ATP production of intact muscle mitochondria [22•] in patients with PD.

Blood lactate and pyruvate levels were reported to be normal in 23 patients with PD at rest as compared with 14 control subjects [23] Blood lactate levels were also unchanged following a glucose load [12] and aerobic exercise [18] Cerebrospinal fluid lactate and pyruvate levels were found to be normal in nine patients with PD as compared with 110 control subjects [18].

To address the question of whether the decrease of complex I activity in the substantia nigra is a primary event in the pathogenesis of PD or whether this biochemical change is secondary to other pathologic processes, Dexter *et al* [24••] examined brain tissue from normal individuals with incidental Lewy bodies and cell loss in pigmented substantia nigra neurons (asymptomatic PD) Complex I activity in the substantia nigra was reduced to levels that were intermediate between those in control subjects and those in patients with overt PD, but this change (29%) did not reach statistical significance The levels of reduced glutathione in substantia nigra were significantly reduced by 35% in patients with incidental Lewy body disease compared with control subjects. The importance of oxidative stress in mitochondrial damage has also been shown in studies of glutathione depletion Exposure of brain to oxidative stress leads to a marked depletion of reduced glutathione [25] These findings suggest that oxidative stress is an early and important change in the pathogenesis of PD

The high rate of spontaneous mutations and the poor repair capacity of mitochondrial DNA lead to a gradual accumulation of mutations, especially in postmitotic tissue such as heart, muscle, and brain. The common 4977 nucleotide pair deletion (common deletion) between nucleotide 8470 and nucleotide 13447 of the mitochondrial DNA was reported to be increased in elderly individuals Deletion levels were especially high in muscle tissues and in all brain regions tested [26] Furthermore, the ratio of deleted mitochondrial DNA to normal mitochondrial DNA was found to be several hundredfold higher in substantia nigra, caudate, putamen, and pallidum than in the cerebellum, being highest in substantia nigra [27,28]. In PD, initial studies using polymerase chain reaction suggested that the common deletion was considerably more common in the striatum of patients with PD than in age-matched controls [29,30]. However, more recent studies have not shown any increase of mitochondrial deletions in the substantia nigra, putamen, cortex, or muscle of patients with PD [17,20•,31–34]. Recently, Shoffner *et al.* [35•] showed several point mutations and one insertion with increased frequency in patients with combined Alzheimer's disease and PD, which may serve as a risk factor for these diseases

It was questioned whether the complex I deficiency observed in PD may be, at least in part, secondary to drug therapy. Due to structural similarities with MPTP and the ability to cause a parkinsonian syndrome, Burkhardt *et al.* [36•] studied the effect of neuroleptics on activities of the electron transport chain Haloperidol, chlorpromazine, and thiothixene dose-dependently blocked the activity of complex I *in vitro*. Additionally, complex I activity in platelet mitochondria from patients undergoing psychiatric treatment who were treated with neuroleptics was decreased to 42% of control subjects with the same disease but without medication The medication history, therefore, should be evaluated carefully when measuring electron transport chain enzymes

It was recently shown that chronic L-dopa administration at high doses causes a significant reduction in complex I activity in rats The L-dopa–induced decrease of complex I activity was reversible [37•] Because almost all patients with PD are chronically treated with L-dopa, the authors suggested that this mechanism may exaggerate a mitochondrial defect already present in the brains of patients with PD and,

thus, may play a role in the progression of PD. However, other groups were not able to find any difference in the electron transfer chain activities [12,38] Furthermore, patients with MSA who are treated with comparable doses of L-dopa show no decrease of complex I activity either in the substantia nigra postmortem [5] or in platelets [15•].

Multiple system atrophy

Multiple system atrophy is a sporadic disease with onset after the age of 30 years, which encompasses the pathologies of striatonigral degeneration, olivopontocerebellar atrophy, and Shy-Drager syndrome. The most frequent motor symptom is parkinsonism with akinesia and rigor During life it may be difficult to separate MSA from PD Usually, the response to L-dopa treatment in MSA is either poor or less prominent than that in PD. The substantia nigra almost always shows a loss of dopaminergic pars compacta neurons, which can be as severe as that in PD Pathologically, a definite diagnosis of MSA is made by the specific pattern of cell loss and the detection of argyrophilic cytoplasmic inclusion bodies.

Although MSA shares clinical features and loss of substantia nigra neurons with PD, complex I activity is normal in patients with MSA [5]. This normal activity indicates that the complex I defect in PD is not a consequence of cell loss. Schapira *et al* [5] reported a significant decrease of 53% in the activity of cytochrome *c* oxidase (complex IV) in the substantia nigra of patients with MSA. Unfortunately, they did not include patients with MSA in their study of other brain areas Benecke *et al.* [15•] did not find any significant decreases in the activities of the electron transport chain enzymes in the platelets of patients with MSA The question of whether a decrease of complex IV activity is a specific primary defect in the substantia nigra of patients with MSA or a secondary effect following degeneration of neurons remains unclear

Progressive supranuclear palsy

Progressive supranuclear palsy is a degenerative disease characterized by widespread neuronal loss in the brainstem and basal ganglia. The diagnostic clinical feature is an early development of difficulty in voluntary vertical eye movements. Other manifestations of the disease include pseudobulbar palsy, postural instability, bradykinesia, and axial rigidity

Recently, Di Monte *et al* [39•] reported a decrease in muscle mitochondrial ATP production in patients with progressive supranuclear palsy By using different substrates for ATP production, the observed changes did not seem to be due to a specific defect of one of the electron transport chain complexes. It appeared that a loss of the electron transport chain function may particularly affect patients in the more advanced stages of the disease, and therefore may be, at least in part, a consequence of their chronic motor disability Studies of brain tissue will be desirable to determine whether or not a mitochondrial defect is present in affected brain tissue

Huntington's disease

Although the HD gene mutation, an expansion of a $(CAG)_n$ triplet repeat sequence, has recently been found, the function of the gene product is still unknown [40] As shown by *in situ* hybridization, the HD transcript is expressed in neurons throughout the brain in both rats and humans, without pronounced regional differences [41] The regional specificity of neuropathology in HD, which is most prominent in the basal ganglia, thus, cannot be accounted for by the pattern of expression of the HD gene product Several lines of evidence suggest that a fundamental defect in energy metabolism could play a role in the pathogenesis of HD Positron-emission tomography studies of brain energy metabolism show that both glucose and oxygen metabolism are decreased in the caudate and in the cerebral cortex of patients with HD [42–45] Using localized ^1H nuclear magnetic resonance spectroscopy, cortical lactate concentrations were increased in all symptomatic patients with HD who were examined, but not in two asymptomatic individuals with a HD diagnosis made by genetic testing [46••]. Oral treatment of the symptomatic patients with HD with coenzyme CoQ_{10}, which serves as an electron donor and acceptor in the electron transport chain and acts as an antioxidant, led to a significant decrease in brain lactate concentrations in 13 of 15 patients [47].

Biochemical studies in HD postmortem brain tissue and in platelets show defects in mitochondrial energy metabolism [48–50]. Brennan *et al* [48] reported reduced cytochrome aa_3 and cytochrome *c* oxidase activity (complex IV) in caudate nucleus Mann *et al* [49] found decreased complex II and III activity in caudate, whereas complex I and IV activities were in control ranges. Parker *et al.* [50] found decreased complex I activity in platelets

The most compelling evidence that a mitochondrial dysfunction may be involved in the pathogenesis of HD has been the observation that 3-nitropropionic acid produces selective basal ganglia lesions and delayed dystonia when accidentally ingested by humans [51]. 3-Nitropropionic acid is an irreversible inhibitor of succinate dehydrogenase, which inhibits both the Krebs cycle and complex II of the mitochondrial electron transport chain Administration of this mitochondrial toxin in rats results in age-dependent striatal excitotoxic lesions as a consequence of an impairment of mitochondrial energy metabolism [52•,53••]. Chronic administration of 3-nitropropionic acid over 1 month produces selective striatal lesions, which replicate many of the characteristic histologic and neurochemical features of HD [53••] Systemic administration of 3-nitropropionic acid to nonhuman primates (*Macaca nemestrina*) causes a movement disorder that closely resembles HD, with dystonia, choreiform movements, and severe loss of muscle tone [54•] The histologic alter-

ations mimic the profile of neuronal degeneration observed in HD and suggest that the genetic abnormality of HD may be associated with a defect in mitochondrial energy metabolism

Idiopathic dystonia

Segregation analysis has suggested the presence of an autosomal gene or multiple genes with reduced penetrance as a common cause for dystonia, regardless of whether its distribution is generalized or segmental [55] In contrast with PD and HD, which show characteristic neuronal degeneration, dystonia has not been associated with clinical or pathologic evidence of neuronal abnormalities The first, and so far only, biochemical disturbance is a significant decrease of complex I in the mitochondrial fraction of platelets [56] The severity of the complex I defect was more pronounced in patients with the segmental or generalized form than in those with focal dystonia. The activities of the other electron transfer complexes were normal To investigate whether this decrease of complex I activity is a consistent finding and can explain dysfunction of basal ganglia neurons, it will be desirable to investigate postmortem basal ganglia tissue of patients with idiopathic dystonia.

Conclusions

The finding of a progressive age-related accumulation in oxidative damage to DNA in the human brain, which preferentially affects the mitochondrial DNA [57••], indicates a decline of mitochondrial function with aging There are age-related decreases in the activities of complexes I and IV [58•] and in the rate of ATP production [59•] in the brains of primates. It is likely that declines in mitochondrial function contribute to the striking age dependency of neurodegenerative diseases. Furthermore, certain point mutations of the mitochondrial DNA may serve as risk factors for neurodegenerative diseases [35•] The increase of mitochondrial DNA deletions with advancing age, which is highest in the basal ganglia and, in particular, the substantia nigra [27,28], suggests that the basal ganglia may be preferentially vulnerable to an age-related decline in mitochondrial function.

Smith *et al* [60] recently found a 24% decrease in complex I activity of smokers in platelet mitochondria. This result is the first *in vivo* demonstration of mitochondrial inhibition caused by a common environmental agent. It supports the theory that environmental toxins may contribute to impaired mitochondrial function. Exposure to certain environmental toxins in combination with a genetic predisposition due to mutations of mitochondrial DNA may therefore serve as risk factors for neurodegenerative diseases The apparent sporadic inheritance of most cases of PD could be related to this type of pathogenesis. One could envision a mitochondrial DNA mutation that leads to mild impairment of oxidative metabolism and that may be variably expressed due to heteroplasmy. Such a mutation in combination with other genetic and environmental factors, as well as age-related impairment of mitochondrial function, could directly contribute to the pathogenesis of neurodegenerative diseases.

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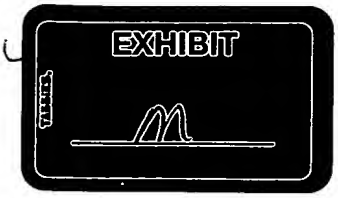

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Neuroprotective Effects of Creatine in a Transgenic Mouse Model of Huntington's Disease

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Huntington's disease (HD) is a progressive neurodegenerative illness for which there is no effective therapy. We examined whether creatine, which may exert neuroprotective effects by increasing phosphocreatine levels or by stabilizing the mitochondrial permeability transition, has beneficial effects in a transgenic mouse model of HD (line 6/2). Dietary creatine supplementation significantly improved survival, slowed the development of brain atrophy, and delayed atrophy of striatal neurons and the formation of huntingtin-positive aggregates in R6/2 mice. Body weight and motor performance on the rotarod test were significantly improved in creatine-supplemented R6/2 mice, whereas the onset of diabetes was markedly delayed. Nuclear magnetic resonance spectroscopy showed that creatine supplementation significantly increased brain creatine concentrations and delayed decreases in *N*-acetylaspartate concentrations. These results support a role of metabolic dysfunction in a transgenic mouse model of HD and suggest a novel therapeutic strategy to slow the pathological process.

Key words: creatine; mitochondria; Huntington's disease; transgenic mice; diabetes; N-acetylaspartate

Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disease that starts in midlife and inexorably leads to death. The mean survival after onset is 15–20 years, and at present there is no known effective treatment for HD. The mutation that causes the illness is an expanded CAG/polyglutamine repeat stretch that has been postulated to confer toxic effects by several different mechanisms (The Huntington's Disease Collaborative Research Group, 1993). The protein product of the HD gene, huntingtin, is expressed ubiquitously in both the nervous system and peripheral tissues (Strong et al., 1993; Landwehrmeyer et al., 1995; Sharp et al., 1995; Ferrante et al., 1997).

A breakthrough in HD research was the development of transgenic mouse models. Transgenic mice expressing exon 1 of the human HD gene with an expanded CAG repeat develop a progressive neurological disorder (Mangiarini et al., 1996). These mice (line R 6/2) have CAG repeat lengths of 141–157 (normal, <35), under the control of the human HD promoter. At ~6 weeks of age the R6/2 mice show loss of brain and body weight, and at 9–11 weeks they develop an irregular gait, abrupt shuddering, stereotypic movements, resting tremors, and epileptic seizures. The mice show an early decrease of several neurotransmitter receptors (Cha et al., 1998). The brains of R6/2 mice appear normal in most respects, however, neuronal intranuclear inclusions that are immunopositive for huntingtin and ubiquitin are detected in the striatum at 4.5 weeks (Davies et al., 1997). Neuropil, cytoplasmic, and neuronal inclusions are also found in human HD (DiFiglia et al., 1997; Gutekunst et al., 1999; Kuemmerle et al., 1999).

A secondary effect of the gene defect may be impaired energy metabolism that may contribute to neuronal death. Consistent with this hypothesis, we and others found that: (1) lactate is elevated in the cerebral cortex and basal ganglia of patients with HD, (2) there is reduced phosphocreatine/inorganic phosphate in resting muscle of HD patients, (3) mitochondrial toxins produce selective damage in the striatum of animals, which closely resembles the pathology of HD, and (4) there are reductions in mitochondrial electron transport enzymes in HD postmortem tissue (Jenkins et al., 1993; Brouillet et al., 1995; Gu et al., 1996; Browne et al., 1997; Koroshetz et al., 1997). Recent studies show increased susceptibility of mitochondria to depolarization in HD lymphoblasts and fibroblasts (Gutekunst et al., 1996; Sawa et al., 1999). Our studies in R6/2 mice show that they develop marked decreases in N-acetylaspartate (NAA) concentrations before neuronal loss (Jenkins et al., 2000) and increased vulnerability to the mitochondrial toxin 3-nitropropionic acid (Bogdanov et al., 1998). This may be a consequence of mitochondrial dysfunction (Bates et al., 1996).

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Table 1. Effects of oral administration of creatine on survival in R6/2 mice

	Unsupplemented R6/2 mice	1% Creatine-supplemented R6/2 mice	2% Creatine-supplemented R6/2 mice	3% Creatine-supplemented R6/2 mice
Days	97.7 ± 0.7	106.6 ± 0.5*	114.6 ± 0.9*	101.9 ± 1.0**

Oral administration of 1, 2, and 3% creatine in the diet dose-dependently improved survival in R6/2 transgenic mice. Oral administration of 2% creatine was significantly greater than 3% creatine ($p < 0.0001$). *$p < 0.0001$; **$p < 0.0002$.

If mitochondrial impairment plays a role in neuronal dysfunction in the R6/2 mice, then buffering intracellular energy levels may ameliorate the neurodegenerative process in these animals. Creatine kinase and its substrates creatine and phosphocreatine constitute an intricate cellular energy buffering and transport system connecting sites of energy production (mitochondria) with sites of energy consumption (Hemmer and Wallimann, 1993). Creatine administration increases brain concentrations of PCr and inhibits activation of the mitochondrial permeability transition (MPT), both of which may exert neuroprotective effects (Hemmer and Wallimann, 1993; O'Gorman et al., 1996). We previously showed that creatine administration is neuroprotective in mitochondrial toxin models of HD (Matthews et al., 1998). In the present study, we examined whether creatine administration exerts beneficial effects on survival as well as the behavioral and neuropathological phenotype in R6/2 mice.

MATERIALS AND METHODS

Transgenic HD mice of the R6/2 strain and littermate controls were obtained from Jackson Laboratories (Bar Harbor, ME). The male R6/2 mice were bred with females from their background strain (B6 CBAF1/J). The offspring were genotyped by PCR assay of DNA obtained from tail tissue. CAG repeat length, using a PCR radioassay method (Wheeler et al., 1999), was examined to ensure that a drift in number of CAG repeats did not play a role in the outcome of the studies (courtesy of Dr. Marcy MacDonald, Massachusetts General Hospital). The repeat length remained stable within a 151–154 range. Transgenic mice were housed in microisolator cages in a modified barrier facility. A 12 hr light/dark cycle was maintained, and animals were given *ad libitum* access to food and water. Groups ($n = 25$) of transgene negative and positive R6/2 mice from the same "f" generation were placed on either unsupplemented diets or diets supplemented with 1, 2, or 3% creatine at 21 d of age (Avicena Group, Cambridge, MA). Approximately 200 mice were used in the survival studies.

Behavioral testing (rotarod). Mice were given 2 d to become acquainted with the rotarod apparatus (Columbus Instruments, Columbus, OH). Testing commenced on day 23. Mice were placed on a rod that was rotating at 10 rpm. Each mouse was given three trials for a maximum of 180 sec for each trial. The length of time at which the mouse fell off the rotating rod was used as the measure of competency on this task. Mice were tested twice weekly until the R6/2 mice were unable to perform the task.

Body weights. Mice were weighed twice a week at the same time of day.

Survival. Mice were observed twice daily, in the morning and late afternoon. The criterion for killing was the point in time when the mice were unable to initiate movement after being gently prodded for 10 min. Two independent observers confirmed this criterion, and this point was used as the time of death.

NMR spectroscopy. We performed *in vivo* spectroscopy at 4.7 T (GE Omega CSI; GE, Freemont, CA) using a sinusoidal bird cage coil (diameter, 20 mm). Mice were anesthetized using halothane/N_2O/O_2 anesthesia (1.5% halothane; 2:1 O_2/N_2O). Body temperature was maintained using two water blankets surrounding the body at 38°C. Localized proton spectroscopy was run using a PRESS sequence (Bottomley, 1987), with an echo time (TE) of 136 msec and a repetition time (TR) of 2 sec. Spectral width was 2 kHz with 1024 complex points. Six hundred averages were acquired for each spectrum. The transmitter frequency was placed between the NAA and creatine resonances. Voxels were placed symmetrically around both basal ganglia with an average voxel size of 6 × 3.5 × 3 μm (63 μl). Spectra were analyzed using the NMR1 software program (New Methods Research, Syracuse, NY). Spectra were integrated, and the choline peak was normalized to the signal-to-noise of a water spectrum run from the same voxel without water suppression, but with a TR/TE of 10,000/20 msec and eight averages. The NAA and total creatine values were then taken as a ratio to the choline peak.

Histological evaluation. At 21 d, R6/2 transgenic mice and negative littermate controls were fed 2% creatine-supplemented and unsupplemented diets. Groups of 20 animals were deeply anesthetized and then transcardially perfused with 4% buffered paraformaldehyde at 21, 28, 42, 63, and 90 d. The brains were removed, post-fixed with the perfusant for 2 hr, weighed, cryoprotected in a graded series of 10 and 20% glycerol/2% DMSO solution, subsequently serially frozen sectioned at 50 μm, stored in six well tissue collection clusters, and stained for Nissl substance (cresyl violet). Serially cut tissue sections were immunostained for huntingtin using an antibody (EM48; dilution, 1:1,000) that recognizes the first 256 amino acids of human huntingtin lacking the polyglutamine and polypeptide stretches (courtesy of Dr. Xiao-Jiang Li) (Gutekunst et al., 1999). The antibody reacts with N-terminal fragments of huntingtin expressed by transfection. It is a sensitive marker of huntingtin aggregation. An antibody to ubiquitin (dilution, 1:200; Dako, Carpinteria, CA) was used in tissue sections to confirm the presence of aggregates.

Stereology and quantitation. Serial-cut coronal tissue sections from the rostral segment of the neostriatum to the level of the anterior commissure (interaural 5.34 mm/bregma 1.54 mm to interaural 3.7 mm/bregma −0.10 mm) (Franklin and Paxinos, 1997), including the primary motor cortex, were used for neuronal and huntingtin aggregate analysis. Unbiased stereological counts of huntingtin-positive aggregates (≥1.0 μm) were obtained from the neostriatum and layer 6 of the neocortex in 10 animals from unsupplemented and 2% creatine-supplemented R6/2 mice at 28, 42, 63, and 90 d using Neurolucida Stereo Investigator software (Microbrightfield, Colchester, VT). The total areas of the neostriatum and motor cortex were defined in serial sections in which counting frames were randomly sampled. The dissector counting method was used in which huntingtin-positive aggregates were counted in an unbiased selection of serial sections in a defined volume of the neostriatum and neocortex. Striatal neuron areas were analyzed by microscopic videocapture using a Windows-based image analysis system for area measurement (Optimas; Bioscan Incorporated, Edmonds, WA). The software automatically identifies and measures profiles. All computer-identified cell profiles were manually verified as neurons and exported to Microsoft Excel. Cross-sectional areas were analyzed using Statview.

Glucose tolerance test. After 6–7 hr of fasting, baseline levels of glucose were measured. The mice were lightly anesthetized with isoflurane gas, and tail vein blood was collected. The mice were subsequently given a bolus injection of glucose (1.5 gm/kg, i.p.), and plasma glucose levels were measured 30 and 60 min later with Lifescan One Touch basic glucose monitoring system (Johnson & Johnson) and validated by semi-automatic glucose oxidase enzyme assay (Beckman).

Statistics. Statistical comparisons for survival were made by the Mantel-Cox log-rank test. Statistical comparisons of other parameters were made by ANOVA or repeated measures ANOVA of other parameters followed by the Fisher's least significant difference test.

RESULTS

The effects of oral administration of creatine in the diet on survival in HD transgenic mice are shown in Table 1. Oral administration of 1% creatine or 2% creatine in the diet dose-dependently improved survival. Administration of 3% creatine significantly improved survival, but was not as effective as either 1 or 2% creatine. Oral administration of 2% creatine was significantly more efficacious than 3% creatine ($p < 0.0001$). The mean survival in controls increased from 97.6 ± 0.7 d to 106.6 ± 0.5 d with 1% creatine ($p < 0.0001$), to 114.6 ± 0.9 d with 2% creatine ($p < 0.0001$), and 101.9 ± 1.0 d with 3% creatine ($p < 0.0002$). The percentage increase in survival for 1, 2, and 3% creatine was 9.4, 17.4, and 4.4%, respectively.

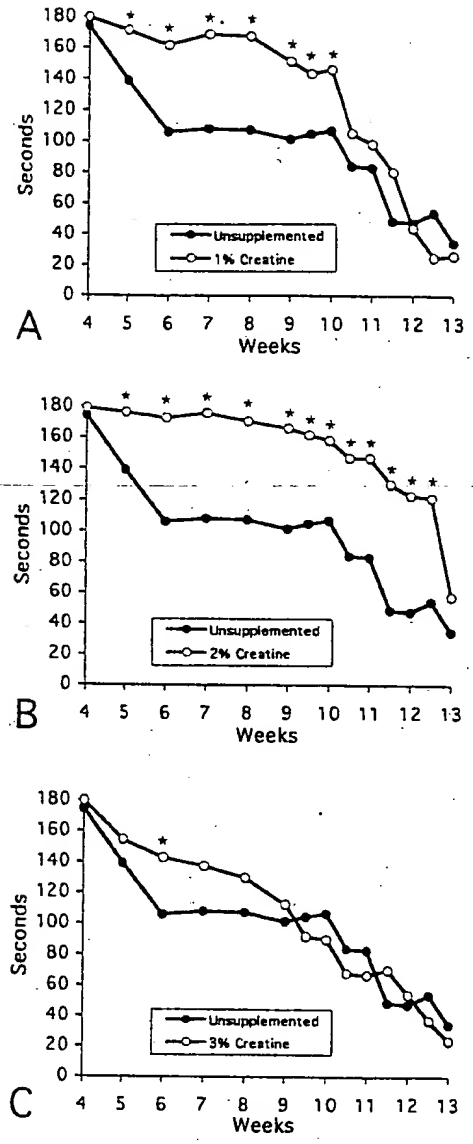

Figure 1. Effects of 1, 2, and 3% creatine on rotarod performance. There was significantly improved performance in R6/2 HD transgenic mice with 2% creatine supplementation throughout the temporal sequence of the experiment (4–13 weeks) (*B*), from 5–10 weeks in 1% creatine-treated mice (*A*), with significance only occurring at 6 weeks in the 3% creatine-treated R6/2 mice (*C*).

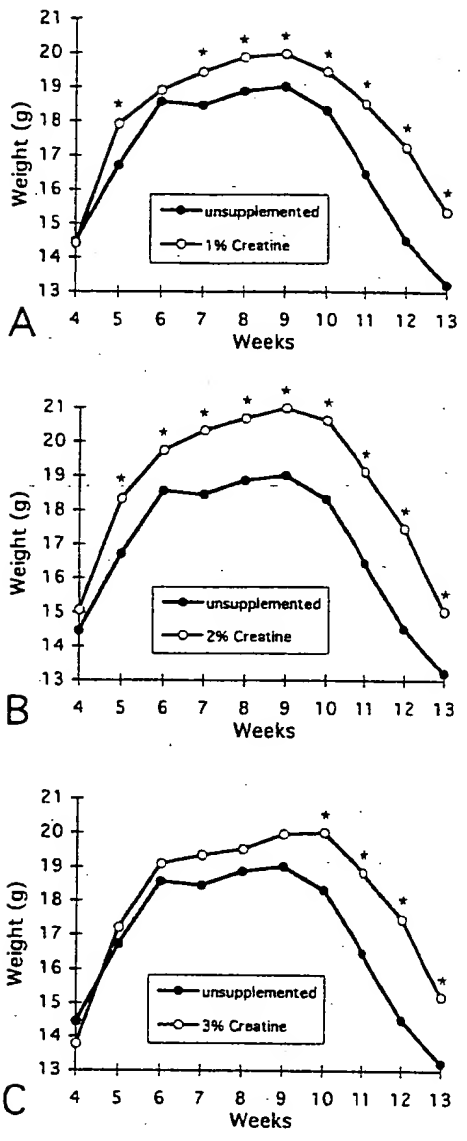

Figure 2. Effects of 1, 2, and 3% creatine on body weight in R6/2 HD transgenic mice. Whereas significantly greater body weight was observed throughout the measured temporal sequence in 1% (except 6 weeks) and 2% creatine-supplemented R6/2 mice, significance was present only from 10 to 13 weeks in the 3% treated mice.

The effects of oral administration of creatine in the diet on rotarod performance between 21 and 90 d are shown in Figure 1. There was a dose-dependent effect of creatine supplementation. Oral administration of 2% creatine significantly improved rotarod performance throughout the entire measured (4–13 weeks) life span of the R6/2 mice in contrast to unsupplemented R6/2 mice (2% creatine, 156 ± 20 sec; unsupplemented, 88 ± 39 sec, $p < 0.001$, data represents combined means and SDs from 5 to 12.5 weeks). Dietary supplementation with 1% creatine resulted in significant motor improvement from 5 to 10 weeks (1% creatine, 161 ± 14 sec; unsupplemented, 114 ± 28 sec, $p < 0.001$, data represents combined means and SDs from 5 to 10 weeks), with no significance observed after 10 weeks. Oral supplementation using 3% creatine had no significant effect on rotarod performance. The percent increase in rotarod performance for 1, 2, and 3% creatine was 25, 33, and 6.5%, respectively.

The effects of oral administration of creatine on body weight in HD transgenic mice are shown in Figure 2. Whereas all creatine regimens resulted in significant improvement of body weight in comparison to unsupplemented R6/2 mice, 2% dietary creatine supplementation resulted in a significantly greater body weight gain in R6/2 mice ($p < 0.01$) than either 1 or 3% creatine supplementation ($p < 0.02$). Significantly greater body weight measurements were present throughout the temporal sequence of measurements (5–13 weeks) in 1 and 2% supplemented R6/2 mice, but were only found from 10–13 weeks in the 3% supplemented group. The total percentage of increase in body weight from 4 to 13 weeks for 1, 2, and 3% creatine-fed R6/2 animals in comparison to unsupplemented R6/2 mice was 6.8, 10.3, and 6.5%, respectively. In comparison to transgene-negative littermate control mice, significant body weight loss in 2% creatine-supplemented mice was delayed until 10 weeks of age. In contrast, significant body weight loss began after 6 weeks in untreated R6/2 mice (data not shown).

Gross brain weights of unsupplemented R6/2 mice decreased significantly over time until death in comparison to both transgene-negative littermate control mice and 2% creatine-supplemented R6/2 mice at all time points (Table 2). By 90 d, there was a 20.4% reduction in brain weight in contrast to littermate control mice. In comparison, there was no

Table 2. Brain weights/2% creatine-supplemented and unsupplemented R6/2 mice

	Unsupplemented R6/2 mice	2% Creatine-supplemented R6/2 mice	Littermate controls mice
28 d	402 ± 5*	414 ± 5	412 ± 4
42 d	395 ± 6*	416 ± 4	424 ± 6
63 d	375 ± 9*	411 ± 8	425 ± 6
90 d	348 ± 13*	407 ± 10**	437 ± 5

Paraformaldehyde-fixed brain weights in milligrams from unsupplemented, 2% creatine-supplemented, and littermate transgene negative R6/2 mice. *$p < 0.002$; **$p < 0.005$.

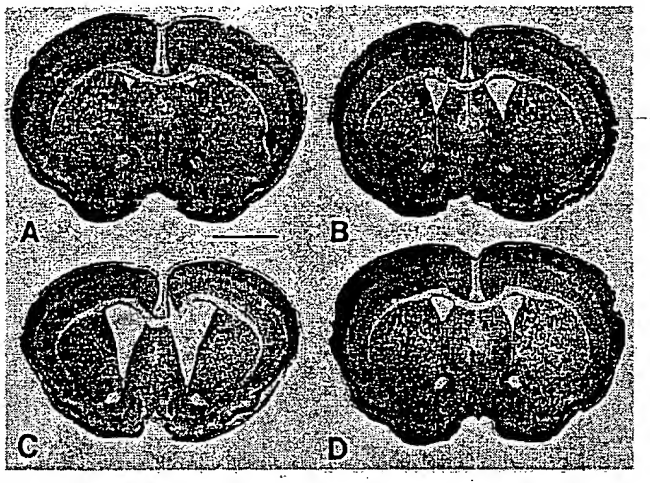

Figure 3. Photomicrographs of coronal sections through the rostral neostriatum at the level of the anterior commissure in R6/2 HD transgenic mice at 42 (*A*), 63 (*B*), and 90 (*C*) d. Note the generalized gross atrophy of the brain over time along with enlargement of the lateral ventricles. In contrast, a 2% creatine-supplemented R6/2 mouse at 90 d (*D*) shows significantly less atrophy and ventricular enlargement than the unsupplemented mouse (*C*). Scale bar, 2 mm.

significant decrease in brain weight in the 2% dietary creatine-supplemented R6/2 mice as compared to controls until end stage measurements at 90 d. At that time there was a 6.8% difference in brain weight (Table 2). Coincident with brain weight loss, progressive marked gross atrophy was present in the unsupplemented R6/2 brains, especially within the neostriatum (Fig. 3). The striatal atrophy was reminiscent of the neuropathological grading we observed in Huntington's disease (Vonsattel et al., 1985), such that there was bilateral ventricular enlargement with a flattening of the medial aspect of the striatum in the late stages of the illness. Dietary 2% creatine supplementation reduced gross brain atrophy in R6/2 mice in comparison to the untreated R6/2 mice (Fig. 3).

Consistent with the gross brain weight loss and striatal atrophy, there was significant progressive atrophy of striatal neurons from 21 to 90 d in the unsupplemented R6/2 mice with a 37.9% overall decrease in area measurements (striatal neurons R6/2 at 28 d, 88.8 ± 10.7 μm^2; striatal neurons R6/2 at 90 d, 55.1 ± 16.8 μm^2; $p < 0.001$) (Figs. 4, 5). The cytoprotective effect of 2% creatine significantly delayed striatal neuron atrophy. There were no significant differences in neuronal areas in 2% creatine-supplemented R6/2 mice until endstage measurements at 90 d of age (Fig. 4).

We examined six mice fed with 2% creatine and eight unsupplemented mice using NMR spectroscopy at 51–57 d of age. As compared with unsupplemented mice, there was a significantly higher NAA/choline ratio in the creatine-fed mice (0.37 ± 0.06 vs 0.48 ± 0.03; $p < 0.05$). The creatine/choline was significantly increased from 0.67 ± 0.04 to 0.83 ± 0.07 ($p < 0.01$). In a total of 13 mice fed creatine compared to 14 mice on unsupplemented diets, including mice older than 70 d of age, the NAA/choline ratio was 0.54 ± 0.03 vs 0.45 ± 0.04 ($p = 0.09$). In the total

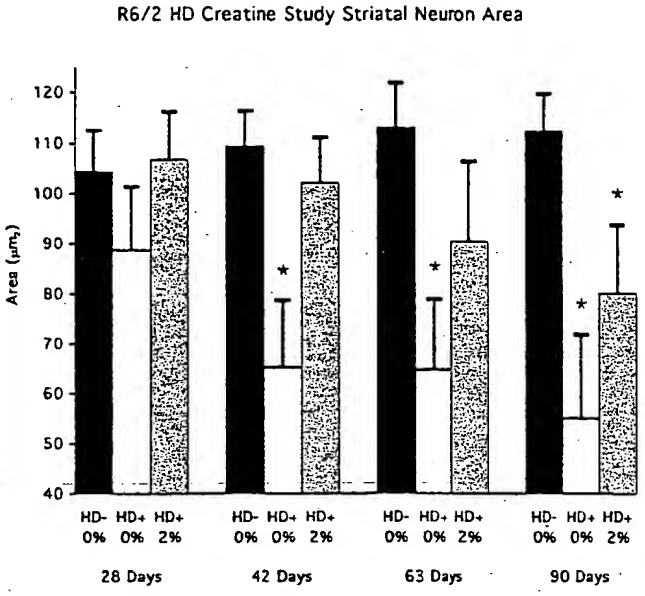

Figure 4. Neuronal areas of 2% creatine-supplemented and unsupplemented R6/2 mice in comparison to littermate transgene-negative mice at 28, 42, 63, and 90 d; *$p < 0.001$.

group the creatine/choline was 0.85 ± 0.04 vs 0.72 ± 0.03 ($p < 0.01$). When normalized to a water standard, there was a significant 21.3 ± 3.8% increase in brain creatine concentrations. In the creatine-treated mice there was a significant correlation between NAA/choline and Cr/choline ($p < 0.01$) that was not seen in the unsupplemented mice (Fig. 6).

An analysis of the formation of aggregates in the neostriatum and cortex of R6/2 mice showed an early and progressive accumulation of huntingtin-positive aggregates, as well as an increase in aggregate size, from 21 d of age to the data collection end point at 90 d. Aggregates were much more prominent within the cortex in comparison to the neostriatum. Dietary supplementation with 2% creatine resulted in a significant reduction in striatal aggregate number throughout treatment at each measured time point (Figs. 7, 8). At 28, 42, 63, and 90 d, the percentage of decrease in aggregate number as compared with unsupplemented mice was 60, 51, 35, and 39%, respectively. A similar trend toward decreased aggregate number was present within the cortex in 2% creatine-treated R6/2 mice, however these decreases were not significant (Figs. 7, 9).

In the unsupplemented R6/2 mice, there was an increase of huntingtin-positive aggregates in the pancreatic Islets of Langerhan over time that was first observed at 42 d and became most prominent at 90 d (Fig. 10). Little or no detectable huntingtin-positive aggregates were observed in the pancreatic stroma. Dietary 2% creatine significantly reduced aggregate number in the pancreas of 90-d-old R6/2 mice (2% creatine, 57 ± 12/field; unsupplemented, 139 ± 15/field; $p < 0.001$) (Fig. 10). Furthermore, administration of 2% creatine significantly delayed the onset of diabetes as assessed by a glucose tolerance test at 8.5 weeks of age (Fig. 11).

DISCUSSION

The development of transgenic mouse models of neurodegenerative diseases provides a major advance for studying disease pathogenesis and for developing therapeutics. If therapeutic effects in the transgenic mice are shown to be predictive of beneficial effects in man, then this will allow rapid screening for new therapies. As discussed above, there is substantial evidence that energy dysfunction occurs in HD, and that this may play a role in cell death. Creatine administration, as discussed below, has several potential neuroprotective effects, including buffering of intracellular energy reserves, stabilizing intracellular calcium, and inhibiting activation of the MPT, all of which have been linked to excitotoxic and apoptotic cell death (O'Gorman et al., 1997; Leist and Nicotera, 1998; Lipton and Nicotera, 1998).

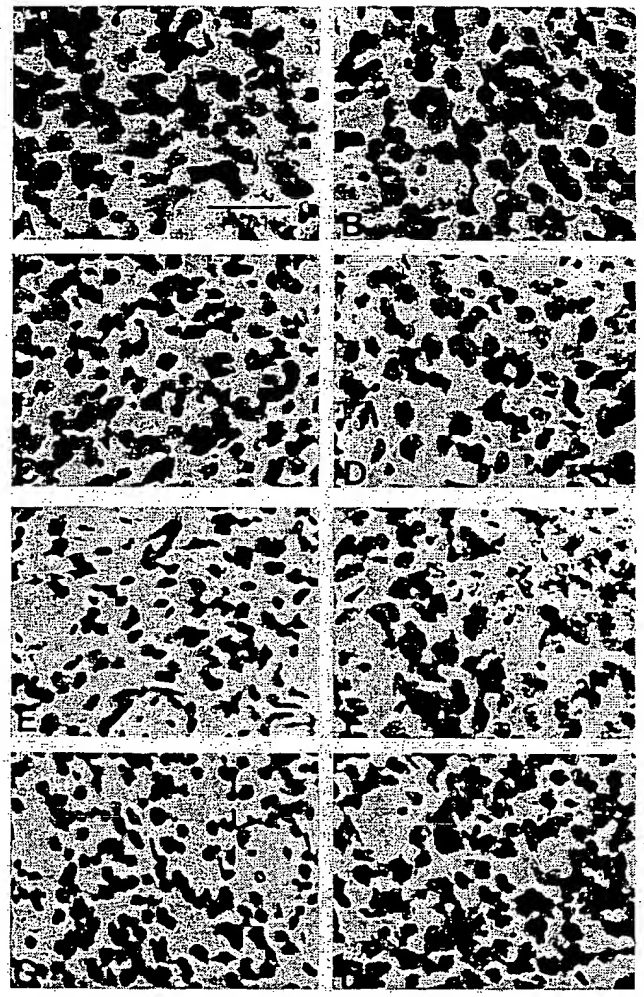

Figure 5. Photomicrographs of Nissl-stained tissue sections from the dorsomedial aspect of the neostriatum (*A, C, E, G*) and 2% creatine-supplemented (*B, D, F, H*) R6/2 HD transgene mice at 4 (*A, B*), 6 (*C, D*), 9 (*E, F*), and 13 (*G, H*) weeks. Note the progressive loss in neuronal size in the unsupplemented R6/2 group, with delayed neuronal atrophy in the 2% creatine-supplemented R6/2 mice. Scale bar, 100 μm.

The brain isoform of creatine kinase along with the mitochondrial isoform and the substrates creatine and phosphocreatine constitute a system that appears to be critical in regulating energy homeostasis in the brain (Hemmer and Wallimann, 1993). There is evidence for a direct functional coupling of creatine kinase with sodium potassium ATPase, neurotransmitter release, maintenance of membrane potentials, and restoration of ion gradients after depolarization (Dunant et al., 1988; Hemmer and Wallimann, 1993). An important role of creatine kinase in the adult brain is supported by *in vivo* ^{31}P NMR transfer measurements. These show that creatine kinase flux correlates with brain activity as measured by the EEG, as well as with amounts of 2-deoxyglucose uptake in the brain (Sauter and Rudin, 1993; Corbett and Laptook, 1994). We previously showed that administration of creatine increases brain phosphocreatine concentrations and buffers against toxin-induced depletions (Matthews et al., 1998).

Creatine kinase also appears to be coupled directly or indirectly to energetic processes required for calcium homeostasis

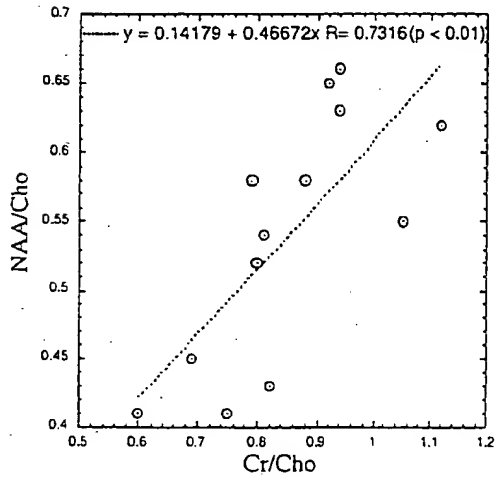

Figure 6. Correlation between creatine and NAA levels in HD transgenic R6/2 mice. Correlation between NAA and CR in unsupplemented mice was not significant.

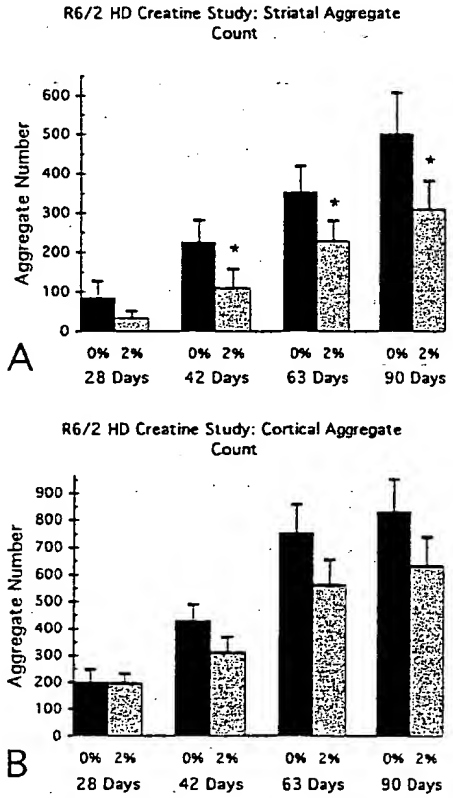

Figure 7. Graphs of the temporal sequence in the number of huntingtin-positive aggregates in the neostriatum (*A*) and motor neocortex (*B*) at 4, 6, 9, and 13 weeks. There was a significant delay in the formation of aggregates within the striatum in 2% creatine-supplemented R6/2 mice, in comparison to unsupplemented R6/2 mice. Although a similar trend was observed in the neocortex, significance was not obtained.

(Wallimann et al., 1992; Steeghs et al., 1997). Creatine pretreatment delays increases in intracellular calcium produced by 3-nitropropionic acid in cortical and striatal astrocytes *in vitro* (Deshpandé et al., 1997). Another potential neuroprotective mechanism is the ability of phosphocreatine to stimulate synaptic

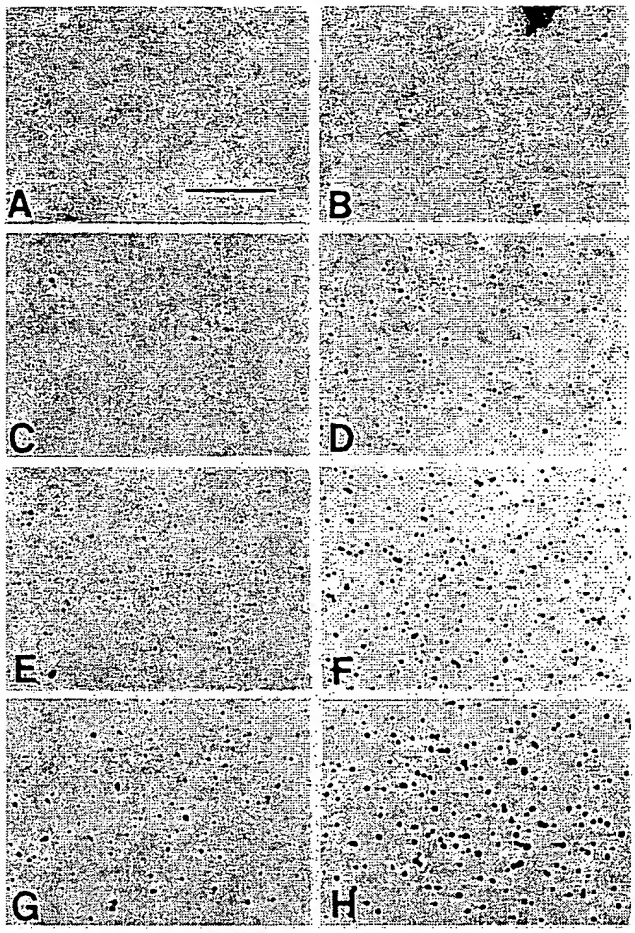

Figure 8. Photomicrographs of huntingtin-immunostained tissue sections from dorsomedial aspect of the neostriatum at the level of the anterior commissure in 2% creatine-supplemented (*A, C, E, G*) and unsupplemented (*B, D, F, H*) R6/2 HD transgene mice at 4 (*A, B*), 6 (*C, D*), 9 (*E, F*), and 13 (*G, H*) weeks. There is a progressive increase in number and size of huntingtin aggregates over time in the unsupplemented R6/2 mice in comparison to the delay in aggregate formation in the 2% creatine-supplemented R6/2 mice. Scale bar, 50 μm.

glutamate uptake and thereby reduce extracellular glutamate (Xu et al., 1996).

Lastly, creatine may protect against activation of the MPT, which is associated with both apoptotic and necrotic cell death (Bernardi et al., 1998). The MPT is a Ca^{2+}-dependent increase of the inner membrane permeability to ions and solutes of up to 1500 Da. Mitochondrial creatine kinase is implicated in a functional interaction between the outer membrane voltage-dependent anion channel and the inner membrane adenylate translocator, which are components of the MPT (Brdiczka et al., 1998). Creatine administration can stabilize mitochondrial creatine kinase in an octomeric form, which inhibits activation of the MPT (O'Gorman et al., 1997). Mitochondrial creatine kinase is also a prime target for peroxynitrite-induced damage, which results in dissociation of mitochondrial creatine kinase octomers into dimers (Soboll et al., 1999). Creatine administration inhibits peroxynitrite-induced modification and inactivation (Stachowiak et al., 1998). Creatine administration may also increase ADP concentrations, which inhibits activation of the MPT (Bernardi et al., 1998).

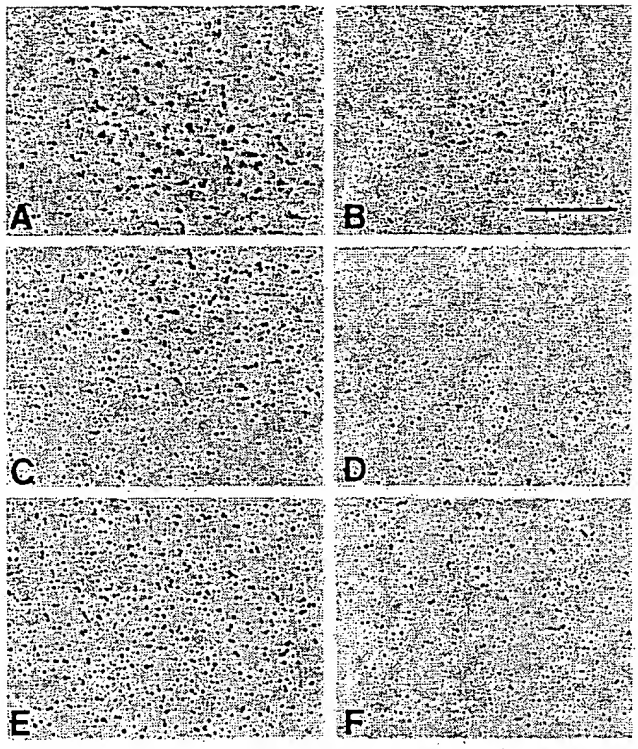

Figure 9. Photomicrographs of huntingtin-immunostained tissue sections from layer 6 of the motor cortex at the level of the anterior commissure in unsupplemented (*A, C, E*) and 2% creatine-supplemented (*B, D, F*) R6/2 HD transgene mice at 4 (*A, B*), 9 (*C, D*), and 13 (*E, F*) weeks. There is a progressive increase in number and size of huntingtin aggregates over time in the unsupplemented R6/2 mice in comparison to the delay in aggregate formation in the 2% creatine-supplemented R6/2 mice. Scale bar, 100 μm.

Previous studies showed neuroprotective effects of creatine *in vitro* and *in vivo*. Creatine reduces anoxic damage to hippocampal slices *in vitro* (Carter et al., 1995). We have found that creatine administration exerts neuroprotective effects against animal models of Huntington's disease produced by administration of the mitochondrial toxins malonate and 3-nitropropionic acid (Matthews et al., 1998). Creatine administration also attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopamine depletions and substantia nigra neuronal loss (Matthews et al., 1999). In addition, creatine administration increases survival and improves motor performance in a transgenic mouse model of amyotrophic lateral sclerosis and results in marked neuroprotective effects against the loss of anterior horn motor neurons and substantia nigra dopaminergic neurons (Klivenyi et al., 1999).

In the present study, we therefore investigated whether creatine could exert neuroprotective effects in a transgenic mouse model of HD. We found that creatine dose-dependently improved survival in these mice. The increased survival results are comparable to the report of Ona et al. (1999) in which R6/2 mice were crossed with mice with a dominant-negative inhibitor of caspase 1. In that study the maximal increase in survival was 20 d or 20%, whereas in our study 2% creatine increased survival by 17 d or 17.5%. The effect with 3% creatine, however, was less than that seen with either 1% or 2% creatine, consistent with an inverted dose–response curve. We have made similar observations with mal-

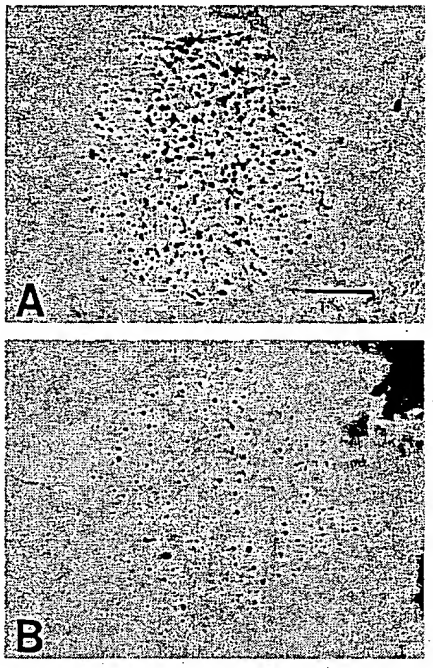

Figure 10. Photomicrographs of islets of Langerhan in the pancreas of 90-d-old unsupplemented (*A*) and 2% creatine-supplemented (*B*) R6/2 HD transgenic mice immunostained with EM48 antibody. There is a marked reduction in the huntingtin aggregates within the treated mouse. Scale bar, 50 μm.

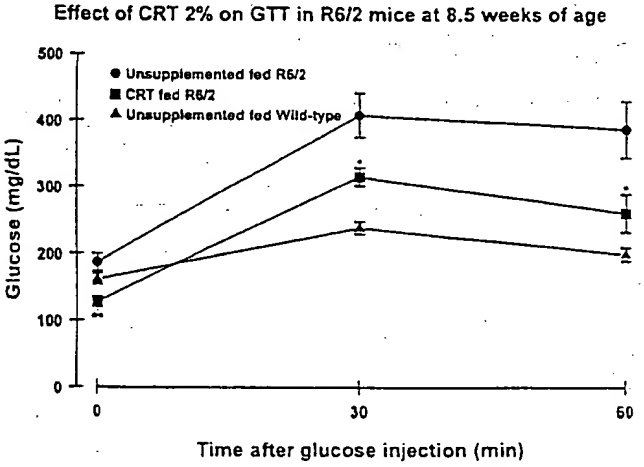

Figure 11. Effects of 2% creatine supplementation on glucose tolerance in 8.5-week-old R6/2 mice. Creatine administration significantly attenuated abnormal glucose tolerance. $^{*}p < 0.05$; $^{**}p < 0.01$.

onate and MPTP toxicity (Matthews et al., 1998, 1999). The explanation for the inverted dose–response curve is unclear, and at very high concentrations creatine may be toxic, similar to observations with cyclocreatine (Matthews et al., 1998).

Creatine administration resulted in improved rotarod performance and reduced weight loss in the R6/2 mice. Similar to effects on survival, 2% creatine was most efficacious, with 1% creatine more effective than 3% creatine. Interestingly, the administration of creatine also delayed the onset of diabetes that has been demonstrated in these mice (Hurlbert et al., 1999). Administration of creatine delayed the development of both striatal and pancreatic huntingtin-positive aggregates, consistent with other recent observations that experimental manipulations can slow the development of nuclear intraneuronal inclusions (Ona et al., 1999). Cross-sectional areas of striatal neurons in R6/2 and other transgenic models of HD have been recently reported to be reduced (Reddy et al., 1998; Hodgson et al., 1999; Levine et al., 1999). Consistent with these findings, we found that administration of 2% creatine significantly delayed the development of both neuronal shrinkage, as well as gross atrophy of the brain. Using NMR spectroscopy we showed that 2% creatine significantly increased brain creatine concentrations by 21% and that it significantly attenuated early decreases in *N*-acetylaspartate concentrations. The increases in creatine peak may largely reflect PCr consistent with our earlier biochemical measurements (Matthews et al., 1998). We recently found a 53% decrease in *N*-acetylaspartate concentrations in the R6/2 mice starting at 6 weeks of age (Jenkins et al., 2000). *N*-acetylaspartate is a neuronal marker that decreases in HD (Jenkins et al., 1998), however it may also reflect mitochondrial function (Bates et al., 1996). NMR spectroscopy could therefore be useful in monitoring therapeutic effects of creatine in patients.

These results provide further evidence that creatine is neuroprotective in animal models of neurodegenerative diseases. Creatine administration is well tolerated in man, results in increased PCr levels, and may have benefits in pathological conditions (Balsom et al., 1994; Dawson et al., 1995; Greenhaff, 1997). Long-term administration to patients with gyrate atrophy of the choroid in the retina prevented visual field constriction and resulted in improvement of muscle biopsy findings (Sipila et al., 1981). Several pediatric patients with creatine deficiency accompanied by an extrapyramidal movement disorder showed partial restoration of cerebral creatine concentrations and clinical improvement after oral creatine administration (Stockler et al., 1994, 1996). Creatine administration has also resulted in significant improvement of patients with the mitochondrial disorder mitochondrial encephalopathy lactic acidosis and strokes (Tarnopolsky et al., 1997), as well as in patients suffering from neuromuscular disorders (Tarnopolsky and Martin, 1999). The present findings support a role for metabolic dysfunction in a transgenic mouse model of HD and provide further evidence that treatment with creatine might be a novel therapeutic strategy to slow or halt the progression of neurodegeneration in HD.

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Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease

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Parkinson's disease is a chronic neurodegenerative disorder characterized by the loss of dopamine neurons in the substantia nigra, decreased striatal dopamine levels, and consequent extrapyramidal motor dysfunction. We now report that minocycline, a semisynthetic tetracycline, recently shown to have neuroprotective effects in animal models of stroke/ischemic injury and Huntington's disease, prevents nigrostriatal dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. Minocycline treatment also blocked dopamine depletion in the striatum as well as in the nucleus accumbens after MPTP administration. The neuroprotective effect of minocycline is associated with marked reductions in inducible NO synthase (iNOS) and caspase 1 expression. *In vitro* studies using primary cultures of mesencephalic and cerebellar granule neurons (CGN) and/or glia demonstrate that minocycline inhibits both 1-methyl-4-phenylpyridinium (MPP^+)-mediated iNOS expression and NO-induced neurotoxicity, but MPP^+-induced neurotoxicity is inhibited only in the presence of glia. Further, minocycline also inhibits NO-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) in CGN and the p38 MAPK inhibitor, SB203580, blocks NO toxicity of CGN. Our results suggest that minocycline blocks MPTP neurotoxicity *in vivo* by indirectly inhibiting MPTP/MPP^+-induced glial iNOS expression and/or directly inhibiting NO-induced neurotoxicity, most likely by inhibiting the phosphorylation of p38 MAPK. Thus, NO appears to play an important role in MPTP neurotoxicity. Neuroprotective tetracyclines may be effective in preventing or slowing the progression of Parkinson's and other neurodegenerative diseases.

Parkinson's disease is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra. The loss of dopaminergic afferents from the substantia nigra to the striatum and putamen results in extrapyramidal motor dysfunction, including tremor, rigidity, and bradykinesia (1). The signs and symptoms of Parkinson's disease can be treated with drugs that increase or enhance dopamine function, but these drugs fail to alter disease progression and most produce undesirable side effects, like motor fluctuations and dyskinesias (2). Several neurotoxins induce Parkinson's-like neuropathology in animals, including the neurotoxins 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (3). MPTP selectively destroys dopamine neurons in the substantia nigra, resulting in a Parkinson's-like syndrome in many species, including humans, monkeys, and mice (4, 5). After parenteral administration, MPTP readily enters the brain and is metabolized by astroglia to 1-methyl-4-phenylpyridinium (MPP^+) (6). MPP^+ is a substrate of the dopamine transporter and is concentrated in nigral dopamine neurons where it inhibits complex I of the mitochondrial electron transport chain, resulting in ATP depletion and subsequent cell death (6). This proposed mechanism of MPTP toxicity implies that dopamine neurons *per se* are the direct cellular targets of MPTP's neurotoxic action.

Recently, however, an important role for glial activation in MPTP neurotoxicity has been suggested by two observations. First, MPTP administration to mice results in a robust gliosis in the substantia nigra pars compacta (SNpc), which is accompanied by up-regulation of inducible NO synthase (iNOS) (7). Second, mice lacking the iNOS or neuronal NO synthase (nNOS) genes are relatively resistant to MPTP toxicity of dopamine neurons compared with wild-type littermates (7–11). Importantly, however, iNOS-deficient mice still manifest a marked reduction in striatal monoamine levels comparable to wild-type controls after MPTP administration (7, 11). These data, coupled with the demonstration that iNOS expression is up-regulated in the substantia nigra of Parkinson's patients, but not from age-matched controls (12), suggest that glial activation and the resulting release of NO (and perhaps other glial-derived neurotoxic substances) may contribute to the chronic neurodegenerative state that characterizes Parkinson's disease.

Minocycline is a semisynthetic second-generation tetracycline that exerts anti-inflammatory effects that are completely separate and distinct from its antimicrobial action (13). Clinical studies have shown that minocycline, and related tetracyclines, have beneficial anti-inflammatory activity and appear to be useful for treating both rheumatoid arthritis and osteoarthritis (14). Tetracyclines, like minocycline, have been reported to have a number of biological and pharmacological actions including an ability to inhibit matrix metalloproteases, superoxide production from neutrophils, and most recently, iNOS expression in human cartilage and murine macrophages (15–17). Minocycline, one of the more brain penetrable of the tetracyclines, has recently been shown to have neuroprotective effects in models of global and focal ischemia (18, 19). The minocycline-induced reduction in infarct size and increased survival of hippocampal neurons after focal or global ischemia, respectively, were accompanied by a reduced expression of IL-1β-converting enzyme (caspase 1), cyclooxygenase-2, and iNOS mRNA in affected brain regions. Furthermore, a recent report by Chen *et al.* (20) demonstrated that minocycline treatment delays mortality in the R6/2 mouse model of Huntington's disease, presumably by

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Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; SNpc, substantia nigra pars compacta; CGN, cerebellar granule neurons; RMN, rostral mesencephalic neurons; iNOS, inducible NO synthase; nNOS, neuronal NO synthase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanilic acid; MAPK, mitogen-activated protein kinase; TH, tyrosine hydroxylase; MAO, monoamine oxidase; SNP, sodium nitroprusside.

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inhibiting caspase 1 and caspase 3 expression, as well as iNOS activity. We now report that oral administration of minocycline to mice effectively blocks MPTP-induced degeneration of dopamine neurons in the SNpc, almost completely preventing the loss of striatal dopamine and its metabilites. Minocycline treatment also inhibits MPP^+-mediated iNOS expression *in vivo* and potently blocks NO-induced neurotoxicity *in vitro*. Thus, indirect and/or direct inhibition of NO-mediated neurotoxicity may underlie minocycline's neuroprotective properties. Minocycline and chemically related neuroprotective tetracyclines may be effective in preventing and/or treating Parkinson's disease.

Materials and Methods

Animals and Treatment. Eight-week-old male C57BL/6 mice (Taconic Farms) were used in all experiments. Mice (5–7 per group) were administered minocycline (60, 90, or 120 mg/kg per day in 5% sucrose; Sigma) by oral gavage before, during, and after MPTP administration. An untreated control group and MPTP-only group were included. The MPTP-treated groups received four injections of MPTP-HCl (20 mg/kg, i.p.) in saline at 2-h intervals in a single day (four injections total) as described (7) and killed at 7 days after the last injection.

Tyrosine Hydroxylase (TH) Immunohistochemistry and Stereological Quantitation of TH-Positive Neurons. After postfixation and cryoprotection in 30% sucrose/phosphate buffer, the brains were frozen in liquid nitrogen and sectioned serially (40 μm) through the entire midbrain. Tissue sections were incubated successively with rabbit polyclonal anti-TH antibody (1:2,500, Calbiochem), goat biotinylated-conjugated polyclonal anti-rabbit antibody (1:250; Vector Laboratories), and horseradish-peroxidase-conjugated avidin/biotin complex (Vector Laboratories). Sections were then exposed to diaminobenzidine for detection. To adequately quantify TH-positive neurons, we used the nuclear counterstain methyl green (Vector Laboratories) and the stereological method for counting TH-positive neurons as described by Triarhou *et al.* (21).

Measurement of Dopamine, 3,4-Dihydroxyphenylacetic Acid (DOPAC), and Homovanilic Acid (HVA) Levels in the Striatum and Nucleus Accumbens. After treatment, the striatum and nucleus accumbens were dissected, frozen on dry ice, and stored at −70°C. HPLC with electrochemical detection was used to simultaneously measure the concentration of dopamine, DOPAC, and HVA in each sample (7, 11, 25).

Measurement of Minocycline and MPP^+ Levels in the Midbrain. Minocycline and MPP^+ were determined in the brain samples by using liquid chromatography with mass spectral detection, which consisted of a Hewlett–Packard model 1100 liquid chromatograph with a Hewlett–Packard model 1946 mass selective detector. A gradient of increasing acetonitrile concentrations in water containing 0.05% trifluoroacetic acid was used to elute the samples from a Zorbax SB-C18, 4.6 × 75-mm column (Hewlett–Packard). The mass spectrometer was run in positive ion mode, fitted with an electrospray ion source, and tuned to select the molecular weights of 171.1 for MPP^+ and 459.9 for minocycline.

Neuronal Cell Cultures and Assessment of Neuronal Viability. Cerebellar granule neurons (CGN) were prepared from 8-day-old Sprague–Dawley rat pups (Harlan Breeders, Indianapolis) as described (22). Primary cultures of rostral mesencephalic neurons (RMN) dissected from embryonic day 15 rat embryos (Harlan Breeders) were prepared as described (23). Cultures were used 2 days after preparation. Neuron/glia cocultures were prepared by modification of a method described by McNaught and Jenner (24). Dopamine neurons in primary cultures were visualized by TH immunohistochemistry (23) and quantified by using a Leitz inverted microscope (×200).

Primary Culture of Astrocyte and Microglia Cells. Briefly, rostral mesencephalic tissue was dissected from embryonic day 15 rat embryos (Harlan Breeders), minced, and incubated in 0.25% trypsin and 0.01% DNase I in PBS for 5 min at 37°C. Cells were resuspended in growth medium then plated in 75-cm^2 flasks coated with poly-D-lysine at a density of 2.0×10^7 cells/flask. Mixed glial cultures were maintained in bicarbonate-buffered DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and passaged twice before use.

Western Blot Analysis. Western blot analysis was performed on brain extracts from selected regions and cell cytoplasmic extracts. Extracts were size-fractionated on a 4–12% polyacrylamide gradient gel (SDS/NuPAGE) and transferred onto nitrocellulose (Hybond N, Amersham Pharmacia). Blots were then probed with polyclonal or monoclonal antibodies, followed by a secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and visualized by using enhanced chemiluminescence.

Results

MPTP-Induced Neurotoxicity of Midbrain Dopamine Neurons Is Blocked by Minocycline. To investigate the neuroprotective effects of minocycline on MPTP-induced dopamine neuronal death *in vivo*, we treated C57BL/6 mice with minocycline (60, 90, and 120 mg/kg orally) daily for 9 days. On day 3, mice were administered MPTP (4×20 mg/kg, i.p.). Seven days after the last dose of MPTP, the brains were analyzed by immunohistochemistry to quantify TH-positive neurons in the SNpc. MPTP treatment reduced the number of TH-positive neurons by ≈63% compared with saline-treated controls ($P < 0.001$) (Figs. 1 and 2). Mice that received daily treatments of minocycline at either 90 or 120 mg/kg, and MPTP showed increased viable TH-positive neurons in the SNpc, ranging from 37% of control (no minocycline treatment) to 56% (90 mg/kg) and 77% (120 mg/kg) of control after minocycline treatment ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 2). The neuroprotective effect of minocycline was dose-dependent as the 60 mg/kg dose of minocycline failed to protect dopamine neurons from MPTP toxicity (Figs. 1 and 2). Minocycline alone did not alters the number of TH-positive neurons significantly.

Minocycline Blocks MPTP-Induced Loss of Striatal Dopamine and Its Metabolites. We next measured striatal levels of dopamine and its metabolites, DOPAC, and HVA by HPLC with electrochemical detection. MPTP treatment reduced striatal dopamine, DOPAC, and HVA levels by 78%, 79%, and 52%, respectively. Minocycline treatment dose-dependently blocked the MPTP-induced decrease in striatal dopamine and dopamine metabolites. Mice that received 90 and 120 mg/kg of minocycline had striatal dopamine levels that were 39% and 83% of untreated controls, respectively, compared with only 22% in the MPTP (alone)-treated group ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 2*B*). Minocycline pretreatment had a similar "protective" effect on striatal DOPAC and HVA levels after MPTP administration (Fig. 2*C*). Consistent with the quantitative data on SNpc dopamine neurons measured by TH immunoreactivity, treatment with 60 mg/kg of minocycline had no significant effect on striatal dopamine or dopamine metabolite levels after MPTP administration. Minocycline pretreatment also blocked the MPTP-induced decrease of dopamine, HVA, and DOPAC in the nucleus accumbens (data not shown).

Minocycline Protects Dopamine Neurons when Administered after MPTP. We next treated animals with minocycline (120 mg/kg, orally) 4 h and 24 h after MPTP administration. Interestingly, minocycline treatment significantly protects against MPTP-induced dopamine neurotoxicity even 4 h after the last (or 12 h after the first) dose of MPTP. Mice that received minocycline beginning 4 h after MPTP treatment showed increased viable TH-positive neurons in the SNpc, ranging from 36% of control (no minocycline

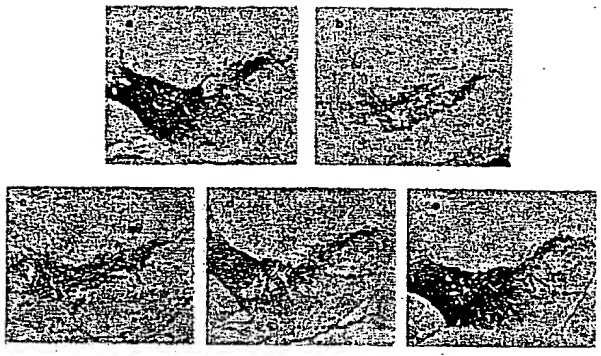

Fig. 1. Minocycline prevents loss of dopamine neurons after MPTP administration. Dopamine neurons and processes were identified by TH immunostaining of representative midbrain sections 7 days after MPTP treatment with or without treatment with minocycline (60, 90, and 120 mg/kg daily, see *Materials and Methods* for details). (*a*) dH_2O. (*b*) MPTP treated. (*c*) MPTP treated after 2 days pretreatment with minocycline 60 mg/kg. (*d*) MPTP treated after 2 days pretreatment with minocycline 90 mg/kg. (*e*) MPTP treated after 2 days pretreatment with minocycline 120 mg/kg. Note the marked reduction in TH-positive cell bodies and processes after MPTP administration (compare *a* and *b*) and the protection by minocycline (*b* vs. *e*). Photomicrographs are from a representative experiment repeated three times with similar results.

treatment) to 66% of control after minocycline (120 mg/kg) treatment ($P < 0.05$). Consistent with the quantitative data on SNpc dopamine neurons measured by TH immunoreactivity, minocycline treatment blocked the MPTP-induced decrease in striatal dopamine. Mice that received minocycline (120 mg/kg) had striatal dopamine levels that were 56% of untreated controls, compared with only 19% in the MPTP (alone)-treated group ($P < 0.01$). Minocycline posttreatment also blocked the MPTP-induced decrease of striatal dopamine metabolites (data not shown). Minocycline treatment, however, failed to protect dopamine neurons when administered 24 h after MPTP administration (data not shown).

Minocycline Does Not Alter Monoamine Oxidase (MAO) Activity Nor Brain MPP^+ Levels. Inhibitors of MAO-B have been found to prevent MPTP-induced neurotoxicity by blocking MPP^+ formation in mouse brain (26). To confirm that the neuroprotective effects of minocycline we observed were not caused by decreased metabolism of MPTP to MPP^+, we evaluated minocycline as an inhibitor of soluble rat brain MAO-A and MAO-B *in vitro* (26). We measured MAO-A and MAO-B activity in the presence and absence of minocycline and found that minocycline did not inhibit MAO-A at concentrations as high as 317 μM and MAO-B at concentrations up to 1 mM. By comparison, the mixed MAO-A and MAO-B inhibitor pargyline inhibited soluble rat brain MAO-A and MAO-B with pI_{50} values of 6.27 μM and 8.19 μM, respectively. Moreover, minocycline treatment had no effect on the concentration of MPP^+ in the midbrain of MPTP-treated mice quantified by liquid chromatography with mass spectral detection. MPP^+ levels in midbrain were 4.2 ± 0.8 μg/g in untreated or 4.8 ± 1 μg/g in minocycline (120 mg/kg)-treated 3 h after MPTP treatment (P = not significant). Furthermore, minocycline did not inhibit [^{3}H]mazindol binding to membranes expressing human dopamine transporters (data not shown). These data suggest that the neuroprotective effect of minocycline is not caused by reduced metabolism of MPTP to MPP^+ or reduced uptake of MPP^+ into dopamine neurons.

Minocycline Blocks MPTP-Induced Expression of Midbrain iNOS and Caspase 1. Because NO synthases and caspase 1 have recently been proposed to mediate (at least in part) MPP^+-induced dopamine neuronal death (7–11, 27) and because minocycline has been shown to inhibit ischemia-induced iNOS and caspase 1 expression in brain

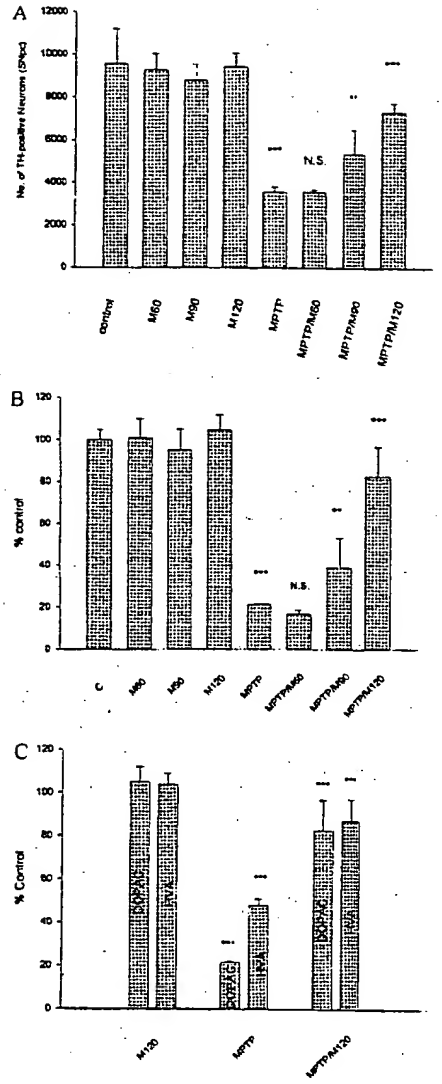

Fig. 2. Minocycline prevents loss of TH-positive neurons, striatal dopamine, and dopamine metabolites after MPTP administration. (*A*) Quantification of TH-positive neurons in the SNpc was carried out as described in the text (21). Minocycline at 90 and 120 mg/kg significantly protects TH-positive neurons from death induced by MPTP exposure (one-way ANOVA; **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant) (see text for details). (*B* and *C*) Dopamine, HVA, and DOPAC were measured by HPLC (see text and ref. 25 for details). Mice administered MPTP showed significant reductions in striatal dopamine, HVA, and DOPAC compared with controls. Minocycline treatment significantly protected animals from MPTP-induced reductions in dopamine, HVA, and DOPAC (one-way ANOVA; **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant). See text for details. Each group consisted of 5–7 animals, and the data are from a representative experiment repeated at least twice with similar results.

(18, 19), we measured both iNOS and caspase 1 in midbrain homogeneates of mice treated with MPTP (Fig. 3). Three to 24 h after MPTP administration, both iNOS and caspase 1 were upregulated in midbrain homogenates as determined by Western blots. Moreover, the latter was blocked by treatment with minocycline (Fig. 3). By contrast, neither MPTP or minocycline had any effect on nNOS expression in these same samples (Fig. 3*A*).

Minocycline Blocks MPP^+-Induced Glial Expression of iNOS and Caspase 1 *in Vitro*. To extend these *in vivo* data, we treated primary cultures of mouse astrocytes and BV2 cells (a mouse microglial

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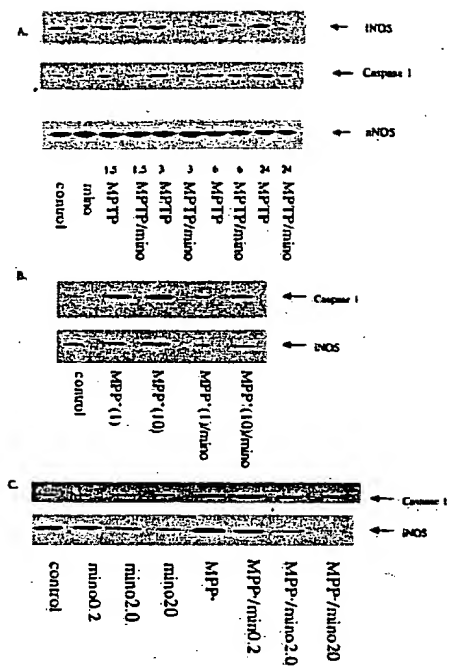

Fig. 3. (*A*) Minocycline blocks MPTP-induced expression of iNOS and caspase 1 *in vivo* and *in vitro*. Immunoblot analyses were performed with polyclonal antibodies against iNOS, nNOS, and caspase 1 (Santa Cruz Biotechnology). Minocycline doses and concentrations as well as the time course after MPTP or MPP^+ administration exposure are indicated. MPTP treatment increases iNOS and caspase 1 expression by 3 h posttreatment. Minocycline treatment blocks the increase in both iNOS and caspase 1. Numbers (i.e., 1.5–24) represent the hours of treatment. Note that MPTP treatment fails to alter nNOS expression in these same samples. (*B*) Minocycline (20 μM) inhibits caspase 1 and iNOS expression induced by MPP^+ (1 and 10 μM, 18 h) in primary cultures of mouse astrocytes. Astrocytes from neonatal mouse cerebral cortex were prepared as described (35). Lane 1 (left to right) = control; lane 2 = MPP^+ (1 μM); lane 3 = MPP^+ (10 μM); lane 4 = MPP^+ (1 μM) minocycline (20 μM); and lane 5 = MPP^+ (10 μM)/minocycline (20 μM). (*C*) Minocycline inhibits caspase 1 and iNOS expression induced by MPP^+ in a mouse microglial cell line (BV2). BV2 cells (36) were cultured to near confluency and then treated with various concentrations of minocycline (0.2–20 μM) with and without MPP^+ (10 μM, 18 h). Note that minocycline reduces basal iNOS expression in BV2 cells and completely blocks iNOS and caspase 1 expression induced by MPP^+.

cell line) with MPP^+, with and without minocycline. Exposure of astrocytes or BV2 cells to MPP+ up-regulates both iNOS and caspase 1 expression as revealed by Western blots (Fig. 3 *B* and *C*). Pretreatment of cultures with minocycline 2 h before MPP^+ treatment dose-dependently reduced MPP^+-induced iNOS and caspase 1 expression in both astrocytes and microglia (Fig. 3 *B* and *C*).

Minocycline Blocks NO, but Not MPP^+-Induced Neurotoxicity in Both CGN and RMN. We next examined whether minocycline could directly block MPP^+-induced toxicity of CGN. CGN represent a relatively homogenous population of neurons that contain ≤5% glia and have been previously shown to be killed by MPP^+ exposure (28). CGN were exposed to MPP^+ (70 μM) in the absence and presence of minocycline (10 and 50 μM), and cell viability was quantified 24 h later. Minocycline treatment had no effect on MPP^+ toxicity of CGN (Fig. 4). Because it has been previously reported that both iNOS and nNOS knockout mice are resistant to MPTP neurotoxicity and that NO is able to potentiate MPP^+-induced dopamine neuronal death *in vitro* (24), we examined whether minocycline could directly block NO-induced neurotoxicity of cultured neurons. Treatment of CGN or RMN with the NO donor sodium nitroprusside (SNP) results in a concentration-dependent cell death (Fig. 4 *A* and *B*). Remarkably, NO-induced neurotoxicity of CGN, was almost completely blocked by minocycline in a concentration-dependent manner ($IC_{50} \approx 1$ μM, Fig. 4*C*).

We next examined the NO-induced loss of dopamine (TH-positive) neurons in primary mesencephalic cultures (Fig. 4 *B* and *D*). Again, SNP treatment (10 μM) induced a ≥80% loss of dopamine neurons and the latter was blocked (≥75%) by minocycline (10 μM) ($P < 0.01$ compared with SNP-treated controls) (Fig. 4*D*). In separate experiments we showed that minocycline (at concentrations ≤10 μM) had no effect on the generation of NO from SNP under our culture conditions (data not shown). As in CGN, MPP^+ toxicity of RMN was unaffected by minocycline treatment (Fig. 4 *B* and *D*).

Minocycline Blocks MPP^+-Induced Neurotoxicity when Assessed in the Presence of Glia. To further test our hypothesis that NO is involved in minocycline's protective effect against MPP^+-induced neurotoxicity, we treated RMN with both subtoxic concentrations of SNP (5 μM) and MPP^+ (0.1 μM) in the absence or presence of minocycline (10 μM). At these concentrations, neither toxin alone resulted in dopamine neuron death, whereas together ≈40% of dopamine neurons were killed ($P < 0.01$), and the latter was completely blocked by minocycline (Fig. 4*E*). Finally, to confirm the postulated role of glia in both MPP^+ neurotoxicity and minocycline-induced neuroprotection, we treated cultures containing both glia and neurons with MPP^+ and minocycline. As predicted, and in contrast to relatively pure neuronal cultures (Fig. 4*B*), minocycline blocks MPP^+-induced dopamine neuronal death in mixed neuron/glia cocultures (Fig. 4*F*).

Minocycline Blocks NO-Induced Phosphorylation of p38 Mitogen-Activated Protein Kinase (MAPK) and an Inhibitor of p38 MAPK Blocks NO Toxicity of CGN. Because Ghatan and colleagues (29) have recently shown that NO-induced apoptosis of neurons is associated with activation of p38 MAPK and that SB203580 (a p38 MAPK inhibitor) blocks NO neurotoxicity, we examined whether minocycline inhibits NO-induced phosphorylation of p38 MAPK in CGN. Pretreatment of CGN with minocycline completely blocks NO-induced p38 MAPK phosphorylation (Fig. 5) without affecting p38 MAPK protein concentration *per se*. Moreover, as previously reported for cultured cortical neurons (29), SB203580 blocks NO toxicity of CGN (data not shown).

Discussion

Our data demonstrate that minocycline can effectively protect midbrain dopamine neurons from the toxic effects of MPTP *in vivo*. Moreover, in contrast to data from iNOS knockout mice (7, 11) minocycline treatment results in a marked "protective effect" on the depletion of dopamine and its metabolites in the striatum and nucleus accumbens after MPTP administration. The neuroprotective effect of minocycline is observed after oral administration even though the oral bioavailability and penetration of minocycline into brain is relatively low in the mouse. However, the oral bioavailability of minocycline and other tetracyclines is considerably higher in humans (30). Both *in vivo* and *in vitro* data demonstrate that minocycline treatment inhibits MPTP/MPP^+-induced iNOS and caspase 1 expression in astroglia and microglia. Because we demonstrate a more robust neuroprotective effect on striatal dopamine levels in minocycline-pretreated mice administered MPTP than that observed in iNOS knockout mice administered MPTP (7, 11), and because minocycline has no effect on nNOS expression (Fig. 3), we examined whether minocycline could directly inhibit NO-mediated neuronal death *in vitro*. We demonstrate that NO-induced neuronal death can be directly blocked by minocycline, and at relatively low concentrations ($IC_{50} \approx 1$ μM). Moreover, the latter correlate with the brain levels of minocycline achieved after oral administration (midbrain minocycline levels were 0.32 ± 0.13 μg/g 8 h after treatment with 120 mg/kg). This finding, coupled with

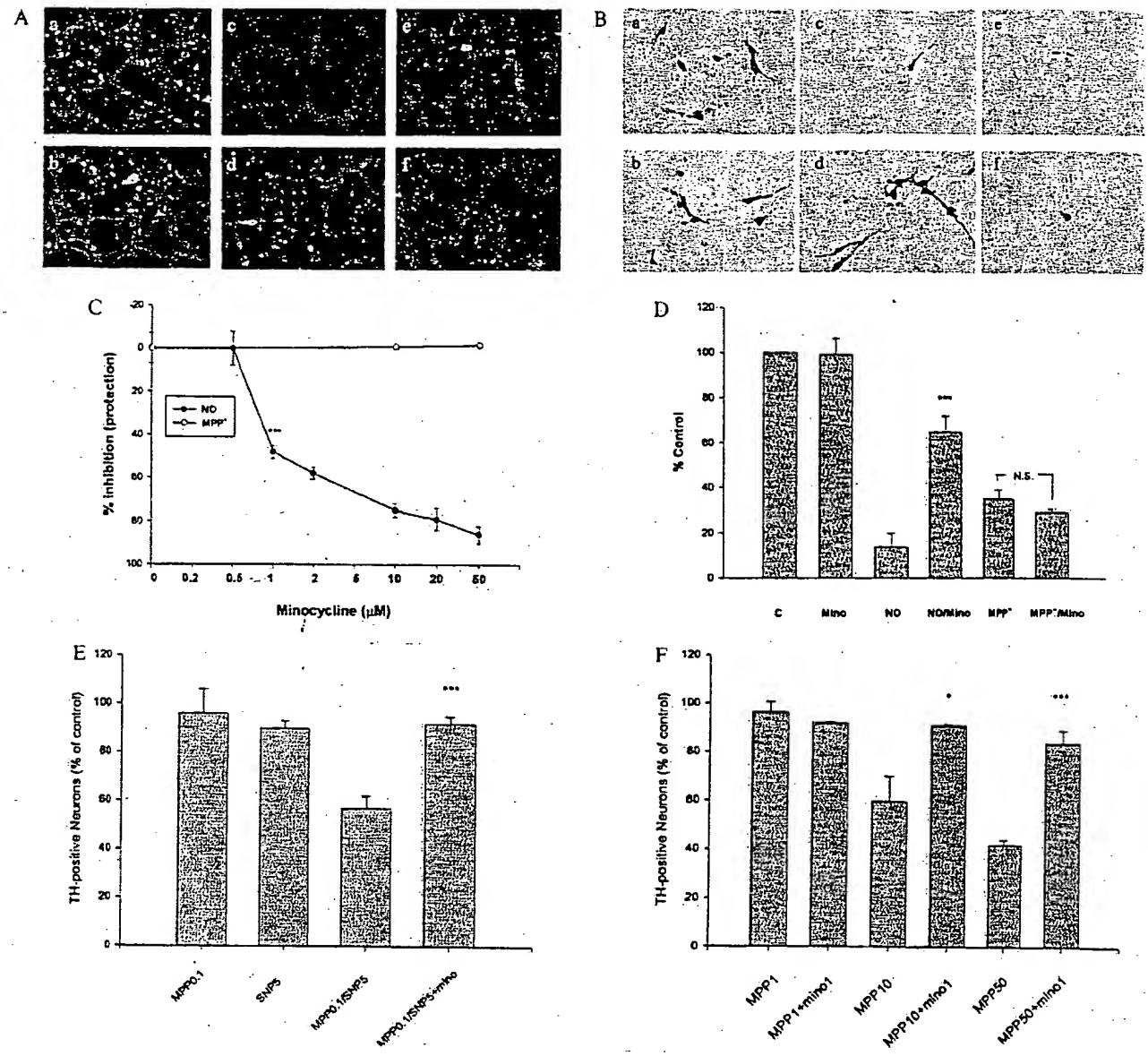

Fig. 4. Effects of minocycline on NO and MPP^+ toxicity of cultured CGN and RMN. (*A*) Minocycline blocks NO-induced neuronal death of CGN, but not MPP^+-induced neurotoxicity. CGN were exposed to increasing concentrations of minocycline (0.5–50 μM) for 24 h in the presence of SNP (50 μM, 24 h) or MPP^+ (70 μM, 72 h). Viable and dead CGN were quantified by using fluorescein diacetate (yellow-green) and propidium iodide (red) staining as described (22). (*a–f*) Representative fields of CGN were photographed (×100) after double staining in the absence (*a*, *c*, and *e*) or presence (*b*, *d*, and *f*) of minocycline (20 μM). (*a* and *b*) No treatment. (*c* and *d*) SNP treatment. (*e* and *f*) MPP^+ treatment. (*C*) Quantification of the effects of minocycline on MPP^+-treated CGN. Values are expressed as a % of control (untreated) cultures for each concentration of minocycline. Data represent the mean ± SE (bars) values of triplicate determinations from a single but representative experiment repeated three times with similar results (•••, $P < 0.001$ by one-way ANOVA; N.S., not significant). (*B*) Minocycline blocks NO-induced neuronal death of cultured RMN but not MPP^+-induced neurotoxicity. (*a–f*) Representative fields of fetal RMN (20) were photographed (×200) after TH staining (see text for details). Compare untreated control and minocycline-treated cultures (*a* and *b*) with those exposed to 10 μM SNP (NO) (*c*) or 10 μM MPP^+ (*e*) plus minocycline (10 μM) (*d* and *f*). Note that minocycline markedly attenuates NO neurotoxicity (*d*), but not MPP^+ neurotoxicity (*f*). (*D*) Quantification of the effects of minocycline on SNP (10 μM) and MPP^+ (10 μM)-treated fetal rat RMN. TH-positive cells were counted from photomicrographs like those shown in *B* above. Data are from a representative experiment repeated twice with similar results (•••, $P < 0.001$ compared with NO alone). (*E*) Minocycline blocks combined NO/MPP^+ toxicity of cultured CGN. Quantification of the effects of minocycline on both SNP (5 μM) and MPP^+ (0.1 μM)-treated CGN. Data are from a representative experiment repeated three times with similar results [•••, $P < 0.001$ compared with SNP (5 μM) and MPP^+ (0.1 μM) alone]. (*F*) Minocycline blocks MPP^+ neurotoxicity in neuron/glia cocultures. Quantification of the effects of minocycline on MPP^+ (1–50 μM)-treated fetal rat RMN/glia cocultures (24). TH-positive cells were quantified from photomicrographs like those shown in *B* above. Note that in the presence of glia higher concentrations of MPP^+ are required to kill dopamine neurons. Nonetheless, in the presence of glia the neurotoxic effects of MPP^+ are completely blocked by minocycline. Data are from a representative experiment repeated twice with similar results. (•, $P < 0.05$; •••, $P < 0.001$ compared with MPP^+ alone).

recent reports on reduced MPTP toxicity in both iNOS and nNOS knockout mice (7–11), or after treatment with NOS inhibitors (8–10), support an important role for NO in mediating MPTP toxicity. Because minocycline does not directly inhibit MPP^+ neurotoxicity *in vitro* in the absence of glia (Fig. 4 *A* and *B*), but does so quite effectively in the presence of glia (Fig. 4 *C* and *D*), we argue that the neurotoxicity of MPTP/MPP^+ observed *in vivo* is mediated (at least in part), indirectly, by NO generated from glial iNOS.

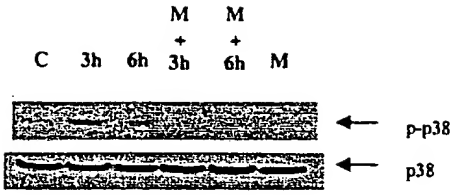

Fig. 5. The effect of NO and minocycline on p38 MAPK phosphorylation in CGN. CGN were exposed to SNP (50 μM) in the absence or presence of minocycline (20 μM) for the indicated times (see text for details). Cell lysates were immunoblotted with anti-phospho-p38 and anti-p38 antibody (New England Biolabs). Note that the increase in phospho-p38 MAPK observed after NO (SNP) treatment is blocked by minocycline (*Upper*). No changes in p38 MAPK itself was observed (*Lower*). Similar results were obtained in three independent experiments. C = control, M = minocycline, p-p38 = phosphorylated p38 MAPK; 3 h and 6 h represent the treatment times of SNP.

We also show that subtoxic concentrations of NO and MPP^+ can kill CGN when combined and that the latter is blocked by minocycline (Fig. 4*C*). It seems quite likely, however, that the dopamine transporter-mediated uptake and concentration of MPP^+ into dopamine neurons, as well as subsequent inhibition of mitochondrial ATP biosynthesis, renders these neurons particularly vulnerable to NO toxicity (24). Synergistic toxic effects of NO (SNP) and MPP^+ were observed in cultured RMN. Thus, we postulate that MPTP neurotoxicity is mediated by both a "direct" (ATP depletion) and "indirect" (NO-mediated) toxic effect on dopamine neurons. Indeed, our data confirm that minocycline is able to block MPP^+-induced dopamine neuronal death in cultures containing both glia and neurons.

Recently, Koistinaho and colleagues (18, 19) have demonstrated neuroprotective effects of minocycline in rodent models of both focal and global ischemia. Infarct size, as well as markers of microglial activation, and the induction of iNOS, cyclooxygenase-2, prostaglandin E2 production, and IL-1β expression were significantly reduced even when minocycline treatment was administered 4 h postinsult. In addition, Chen *et al.* (20) have recently demonstrated a neuroprotective effect of minocycline in the R6/2 transgenic mouse model of Huntington's disease that was associated with inhibition of caspase 1 and 3. Taken together, these reports further suggest that minocycline exerts its neuroprotective effects by "indirectly" inhibiting glial activation and the subsequent release of NO and perhaps cytokines, such as IL-1β (18–20). Although it is likely that such "anti-inflammatory" actions of minocycline undoubtedly contribute to the neuroprotective properties we observe in the MPTP mouse model of Parkinson's disease, our data strongly suggest that minocycline also has a "direct" neuroprotective action as well. Very low concentrations of minocycline are effective in blocking NO toxicity in both CGN and RMN *in vitro* (Fig. 4).

Although the exact cellular mechanism(s) underlying minocycline's direct neuroprotective activity are unknown, we have also found that minocycline inhibits p38 MAPK phosphorylation/activity in CGN (Fig. 5) as well as microglia (data not shown). p38 MAPK, which is activated by a number of cellular "stresses," has recently been implicated in neuronal cell death induced by axotomy (31) and excitotoxicity (32). Moreover, Ghatan and colleagues (29) have shown that p38 MAPK mediates neuronal apoptosis induced by NO, and that p38 MAPK inhibitors block NO toxicity of mouse cortical neurons *in vitro*. Our data, that minocycline treatment of CGN inhibits p38 MAPK activity and that the p38 MAPK inhibitor SB203580 protects CGN from NO toxicity, suggest that inhibition of p38 MAPK may mediate minocycline's direct neuroprotective effects against MPTP/MPP^+ toxicity. Indeed, a very recent report has implicated glial p38 MAPK in the neuroprotective actions of minocycline observed against NMDA toxicity *in vitro* (33). However, our data suggest that minocycline does not directly inhibit p38 MAPK activity but rather inhibits enzyme activation indirectly through reducing phosphorylation, presumably by inhibiting an "upstream" kinase. Additional work will be required to delineate minocycline's exact cellular target(s).

Our findings support an important role for glial activation and NO production in the MPTP model of Parkinson's disease (8–10). Because iNOS expression is up-regulated in the SNpc of Parkinson's patients (12) suggests that a similar mechanism may contribute to the pathogenesis of Parkinson's disease. We caution, however, that MPTP administration, although reliably toxic to dopamine neurons in a variety of species, including humans, may not truly mimic either the etiology or pathophysiology of Parkinson's disease (34). Nevertheless, we also demonstrate that minocycline has robust neuroprotective activity in the MPTP mouse model of Parkinson's disease and provide evidence that this activity is caused by both indirect and direct actions in blocking NO-mediated neurotoxicity. Chemically modified tetracyclines, like minocycline, may prove effective in preventing and/or altering the progression of Parkinson's disease.

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Special Article

Neuroprotective agents for clinical trials in Parkinson's disease

A systematic assessment

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Abstract—*Background:* Current therapies for PD ameliorate symptoms in the early phases of disease but become less effective over time, as the underlying disease progresses. Therapies that slow the progression of PD are needed. However, there have been relatively few clinical trials aimed at demonstrating neuroprotection. The authors sought to identify potential neuroprotective agents for testing in clinical trials. *Methods:* First a broad array of compounds were identified by working with clinicians and researchers in academics and industry. Specific criteria were drafted for drug evaluation, including scientific rationale, blood–brain barrier penetration, safety and tolerability, and evidence of efficacy in animal models or humans. Agents were prioritized based on these criteria. *Results:* The authors identified 59 potential neuroprotective compounds, proposed by 42 clinicians and scientists from 13 countries. After systematic reviews using the specified criteria they found 12 compounds to be attractive candidates for further clinical trials in PD. *Conclusions:* Several potential neuroprotective compounds, representing a wide range of mechanisms, are available and merit further investigation in PD.

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Interventions that can slow or halt the progression of PD remain a crucial unmet need. Few randomized clinical trials have been performed with the primary aim of slowing disease progression in PD, and none have convincingly demonstrated neuroprotection (Appendix 1; go to www.neurology.org). The paucity of clinical trials is due, in part, to a perceived lack of promising interventions.

In order to identify potential neuroprotective agents available for testing in upcoming National Institute of Neurologic Disorders and Stroke (NINDS) sponsored clinical trials in PD (Request for Applications NS-01–012), the Committee to Identify Neuroprotective Agents in Parkinson's (CINAPS) was formed to conduct a systematic assessment of currently available pharmacologic agents. The CINAPS group is composed of experts in PD, clinical trials, and clinical pharmacology. Here, we describe the drug identification and review processes and the most attractive potential neuroprotective candidates for clinical trials.

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the April 22 issue to find the title link for this article.

Need for neuroprotection trials and terms of reference. Existing therapies for PD only temporarily ameliorate symptoms and do not prevent disease progression. In a recent controlled trial, after 2 years of therapy with a dopamine agonist or levodopa, 30–50% of patients begin to experience dopaminergic-related complications, including dyskinesias, wearing off, or on-off motor fluctuations.[1] These motor complications may eventually dominate the clinical picture. Nonmotor symptoms, such as

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depression, dementia, and psychosis, may be unaffected or exacerbated by dopaminergic therapies.[2]

The etiology of PD and its progressive nature are incompletely understood and are most likely multifactorial. The discovery of mutations in alpha synuclein, parkin, and ubiquitin C terminal hydrolase L1 (UCHL-1), associated with hereditary forms of parkinsonism, suggests a common pathway of altered protein processing and degradation that may also contribute to idiopathic forms of the disease.[3,4] Several other processes may initiate or propagate the degenerative process in PD, including oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammation and glial activation, trophic factor deficiency, and finally, apoptosis.[5-7] Environmental insults may trigger or facilitate neurodegeneration.[8,9] The relative contribution of each process in the general PD population is unclear. As in other complex disorders, attenuation of one pathogenic process may lead to incremental clinical improvement, and each individual process may be a valid target for therapies.

We used a broad operational definition of a neuroprotective agent: an intervention that would favorably influence the disease process or underlying pathogenesis to produce enduring benefits for patients.[10] This definition encompasses related terms such as *neurorescue* and *neurorestoration*. The latter terms are useful for discussing interventional strategies and developing therapeutics but clinically may be difficult to distinguish from neuroprotection unless results are dramatic and treated patients actually improve rather than progress more slowly. While the scientific implications differ, the clinical value of a truly enduring symptomatic therapy could be equal to that afforded by a neuroprotective agent. The common goal implicit in all these terms is to slow or halt clinical disease progression regardless of the specific mechanism.

Identification and assessment of potential neuroprotective agents. The initial step of this systematic assessment was to identify potential pharmacologic agents that could be used in trials in the near future. We therefore focused on agents that were at least in the middle stages of development or were already approved for indications other than PD. We did not target surgical therapies or interventions such as trophic factors or stem cells that require surgery for administration. We sought input from scientists and clinicians in academic and industry settings, as well as patient and foundation groups, to capture the broadest range of available compounds (figure). In addition to investigators responding to the National Institute of Neurological Disorders and Stroke-issued request for applications (RFA), we contacted over 100 experts in PD from the United States and 13 foreign countries, 67 pharmaceutical and biotechnology companies, and 10 major US PD advocacy groups. Recommendations of agents for testing were solicited from all researchers funded by National Institute of Neurological Disorders and Stroke and

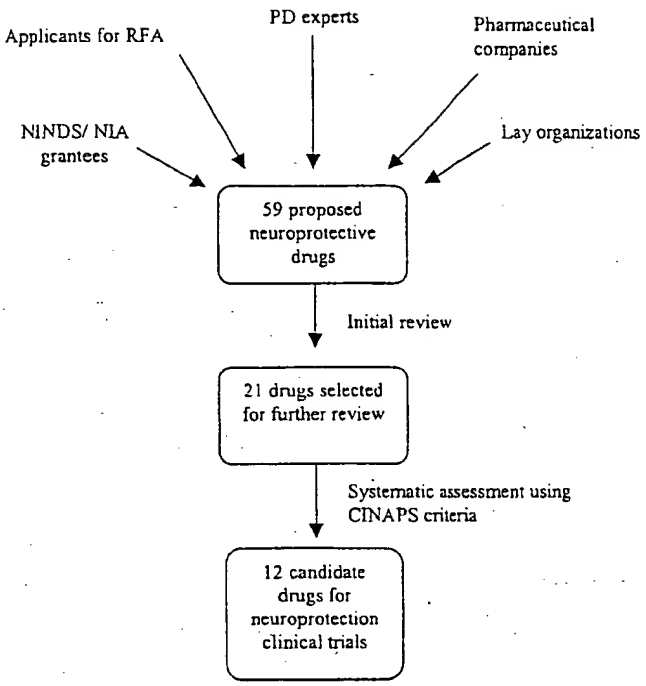

Figure 1. Drug identification and assessment sequence.

NIA during the year 2000, and a request for recommendations was placed on the NINDS Web site. While pharmaceutical and biotechnology companies submitted six investigational compounds, confidentiality agreements prohibit further discussion of five of these agents in this article.

Criteria for drug selection. There are no established criteria for choosing between putative neuroprotective agents that are either validated by clinical trials or supported by a general consensus of experts. Therefore, CINAPS drafted criteria to be used in rating each agent (table 1): scientific rationale, evidence of blood–brain barrier penetration, adequate safety data, and efficacy in animal models and/or preliminary efficacy data in humans. The proposed rating scale was intended to be explicit, transparent, and reproducible, based on a systematic pharmacologic assessment of each agent.

The results of animal models are difficult to compare between agents because of the use of multiple different models and experimental designs. For this reason we focused on methyl-4-phenyl-1, 2,3,6 tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) models. These were the most widely accepted models at the time of the review, providing a useful common denominator. Although both are acute intoxication models with well-known limitations, they replicate features of PD and may have direct validity in our assessment of potential neuroprotective agents. For example, MPTP intoxication kills dopamine neurons, in part, through inhibition of mitochondrial complex I activity and reactive oxygen species production, thus modeling sporadic PD.[11] Relevant data from other animal models, such as transgenic SOD1 rodents, were included.

Table 1 Evaluation criteria for potential neuroprotective drugs

Criteria	Operational definition
Scientific rationale	Consistency of preclinical data; credible mechanism relevant to PD although mechanism may be unknown in many cases
Blood–brain barrier (BBB) penetration	Evidence by direct experiments or inference that BBB penetration occurs and can achieve concentrations needed for the neuroprotective effect with intended route of administration
Safety and tolerability	Safe and tolerable in humans in the dose and route of administration needed for the proposed effect (at least Phase I data); no further safety data required before use in PD
Efficacy in relevant animal models of disease or an indication of benefit in human clinical studies	Animal: consistent efficacy in rodent or nonhuman primate using PD or other relevant model; human: evidence from previous trials that is suggestive of a neuroprotective effect or epidemiologic data fulfilling criteria for causal inference

We limited the clinical data in this review to controlled clinical trials and epidemiologic studies consistent with criteria for causal inference.[12] Neuroimaging studies were not included as evidence of neuroprotection because of their uncertain interpretation.

Systematic pharmacologic assessment of proposed agents. CINAPS conducted PubMed and MEDLINE preliminary literature reviews for all agents to select agents for detailed review and further consideration. Of the 59 drugs identified, we selected 21 for detailed review. More reviews will be conducted in the future; these 21 have been completed as of June 2002. CINAPS chose agents with a broad spectrum of pharmacologic actions, consistent with the many mechanisms thought to be involved in PD. Where multiple agents from the same class were available, we selected the most promising one based on safety or preliminary efficacy. Agents not selected for further review are listed in table 2. Detailed summaries of each drug focusing on the criteria for review were performed by CINAPS, and then each review was returned to the respondent(s) who had initially proposed the drug for comment. CINAPS evaluated these comments and then prepared the final drug summaries, which can be found on the *Neurology* Web site (Appendix 2), as well as the NINDS Web site, http://www.ninds.nih.gov/parkinsonsweb/index.htm. Based on these summaries we rated and prioritized the compounds (table 3). Where there was overlap in class or mechanism, we again chose the representative agent that appeared to be the most

Table 2 Reasons for exclusion of nominated drugs from further review

Agent	Safety concerns*	Mechanism or class covered†	Preliminary search failed‡
Acamprosate		Glutamate antagonist	
AIT-082	x		
Apomorphine		Dopamine agonist	x
Bromocriptine		Dopamine agonist	
(L-acetyl) Carnitine			x
CEP-1347			x
Clioquinol			x
Dimebon			x
Donepezil			x
EGCG (tea extract)			x
KW-6002			x
Gamma-hydroxy butyrate			x
Levetiracetam			x
Lipocortins			x
Lipo-oxygenase inhibitors			x
Lipoic acid			x
Lithium	x		x
Mithramycin			x
7-Nitroindazole			x
Pergolide		Dopamine agonist	
Phenelzine		MAO inhibitor	
Phosphodiesterase inhibitors			x
PolyADP-ribose polymerase inhibitors			x
Resveratrol			x
Riluzole		NMDA antagonist	
Sulforaphanes			x
Taurine			x
Theophylline	x	Adenosine antagonist	
Tocotrienol		Antioxidant	
Topiramate		Glutamate antagonist	
Tranylcypromine		MAO inhibitor	
Uric acid		Antioxidant	
Zonisamide		Antioxidant	

* Human safety concerns relative to other candidates with similar mechanisms.

† Putative mechanism/class covered in top 21.

‡ Preliminary search failed to yield sufficient material for evaluation of rationale and mechanism of neuroprotection, animal or human data supporting neuroprotection or safety and tolerability in humans.

Table 3 CINAPS assessment of 21 potential neuroprotective drugs

	Rationale†				(Preliminary) efficacy¶	
Drug	Primary mechanism	Consistency of preclinical	BBB	Safety/tolerability‡	Animal	Human
Ascorbic acid	Antioxidant	+	+	+	+	−
Amantadine	Glutamate antagonist	−	+	+	+	−
Azulenyl nitrone	Antioxidant	+	+	−	+	−
Caffeine*	Adenosine antagonist	+	+	+	+	+
Coenzyme Q10*	Antioxidant/mitochondrial stabilizer	+	+	+	+	+
COX I–II inhibitors	Anti-inflammatory	−	+	+	+	−
Creatine*	Mitochondrial stabilizer	+	+	+	+	−
Erythropoietin	Undetermined/multiple	+	+	−	+	−
Estrogen*	Undetermined/multiple	+	+	+	+	+
Folate	Undetermined/multiple	−	+	+	+	−
GPI 1485*	Trophic factor	+	+	+	−	−
GM-1 ganglioside*	Trophic factor	+	+	+	+	−
Minocycline*	Anti-inflammatory/anti-apoptotic	+	+	+	+	−
Modafanil	Unknown	+/−	+	+	−	−
N-acetyl cysteine	Antioxidant	+	+	+	+	−
Nicotine	Unknown	−	+	+	+	+
Pramipexole*	Antioxidant/vesicular trafficking	+	+	+	+	+
Ropinirole*	Antioxidant	+	+	+	+	+
Rasagiline*	Anti-oxidant/anti-apoptotic	+	+	+	+	−
Remacemide	Glutamate antagonist	+	+	+	+	−
Selegeline*	Antioxidant/anti-apoptotic	+	+	+	+	+

* Candidate for Phase II or III neuroprotection studies now.
† Primary suspected mechanism(s) explaining neuroprotective effect and consistency of effect in preclinical data.
‡ Such that drug could be given to PD patients now.
¶ Observed in animal studies and suggestion of neuroprotective effect in human clinical trials or epidemiologic studies.

BBB = blood–brain barrier penetration.

promising and had the best supportive data. Through this process we determined that 12 of these drugs are viable candidates to be explored for neuroprotection trials.

Description of priority agents. Caffeine. This ubiquitously available compound may act through adenosine receptor antagonism,[13] although the downstream mechanisms of neuroprotection remain unclear. Prospective epidemiologic data show that caffeine is associated with a reduced incidence of PD,[14] and there are dose-response data to support these observations. There are no published data about rate of progression and caffeine consumption after disease onset, and the observed epidemiologic association may be mediated by another, unmeasured variable. However, animal data from MPTP models support protective effects for caffeine and the more specific A_{2A} receptor antagonist KW-6002.[15,16] The optimal dosage of caffeine is not known. Potential side effects and "cross-contamination" from various sources would be mitigating in a clinical trial setting.

Coenzyme Q 10. This is a commonly used "health supplement" that is being pursued in PD for its ability to augment mitochondrial complex I activity and serve as an antioxidant.[17,18] Doses up to 1,200 mg per day appear safe and well tolerated, and there is some preliminary evidence that this may slow functional decline, as measured by the Unified PD Rating Scale in de novo PD patients.[19]

Creatine. This widely available nutritional supplement has shown promise in a number of neurodegenerative and mitochondrial disorders. Creatine is converted to phosphocreatine, which in turn can function as an energy buffer by transferring a phosphoryl group to ADP. It may act as an indirect antioxidant by enhancing energy transduction and may inhibit mitochondrial permeability transition.[20] In doses of 1 to 2% of diet by weight, creatine appears protective in MPTP rodent models[21] and in transgenic Huntington's disease (HD) and ALS models.[22,23] There is extensive literature on use and tolerability in athletes at doses up to 20 g per day, and there are some data on safety and tolerability in patients with neurologic disorders at doses up to 10 g per day.[20,24]

Estrogen (17 *B* estradiol). The putative neuroprotective effect of this sex hormone is based on epi-

demiologic data on the differences in incidence and progression of PD in men versus women,[25-27] as well as confirmatory animal models of neuroprotection. Estrogens may act through either nuclear transcription events or membrane receptor-mediated signaling.[28,29] Thus the mechanism of neuroprotection is not clear, but may involve neurotrophic effects, synaptic plasticity, or antioxidant effects. Although the drug can be given easily orally, use in men is likely to be associated with unacceptable side effects. Recent data from the Women's Health Initiative testing estrogen and progesterone show an increased risk of cerebrovascular disease and cancer that must be weighed against a potential neuroprotective effect.[30] Nonfeminizing analogs in development that activate desired signal pathways may offer promise.

GM-1 ganglioside. This is an investigational agent, manufactured by Fidia (Abano Terme, Italy), that has been shown to be both neuroprotective and neurorestorative in animal models. It is a ubiquitous component of neuronal membranes that has been proposed to facilitate the neurotrophic actions of brain and glial derived neuroptrophic factors,[31,32] protect against excitotoxicity in culture, and inhibit apoptosis.[33,34] It has been studied extensively in animals, and there are some preliminary data in PD patients suggesting that it is well tolerated and may have short-term symptomatic benefits.[35,36] Its main drawback is the requirement for parenteral administration as well as concerns about the immunogenicity of bovine-derived gangliosides and the relationship to Guillain-Barré syndrome.[37] However, evidence of multiple possible protective mechanisms and data showing that delayed administration is effective, make it an attractive candidate. Orally active, synthetic-derivatives are being explored by US-based pharmaceutical companies.

Minocycline. This is a tetracycline antibiotic with good brain penetration that has been shown to inhibit microglial-related inflammatory events as well as the apoptotic cascade.[38,39]Animal studies in ALS and PD models show convincing evidence of neuroprotection,[40-42] and the compound appears to be safe for chronic administration.[43]

Nicotine. There is remarkably consistent epidemiologic evidence that smokers have a substantially reduced risk of PD compared with nonsmokers.[44,45] It is possible that the protective effect of smoking is not due to nicotine but rather to other components of tobacco smoke. Data on incidence or prevalence, as in the case of caffeine, may not be relevant for disease progression, and the observed association may be due to unmeasured confounders. However, in animals nicotine has been shown to prevent MPTP toxicity. There is evidence that nicotine may act as an antioxidant[46] or prevent excitoxicity.[47] It is unclear whether nicotine can be administered in a way that avoids side effects and dependence in PD patients, and the optimal dosage and method of administration are not known.

GPI-1485. This is an investigational agent, developed by Guilford Pharmaceuticals (Baltimore, MD), that is a neuroimmunophilin ligand. Through interaction with a central FK506 binding protein receptor, GPI 1485 and other neuroimmunophilins demonstrate neurotrophic activity without the immunosuppressive properties of similar compounds, like FK 506.[48] GPI-1485 has been studied in PD patients in a 6-month clinical trial sponsored by Amgen. Data from the trial are published only as a press release, which indicates that the primary endpoint was negative but the drug was well tolerated.

Rasagiline/selegiline. Rasagiline is an investigational selective MAO-B inhibitor (Teva Pharmaceuticals, Israel), related to selegiline but without the amphetamine metabolites. Both rasagiline and selegiline are propargylamines. The mechanism of neuroprotection is likely multifactorial and appears not to be dependent on MAO-B inhibition. There is evidence for an anti-apoptotic effect for this class of agents.[49,50] Rasagiline has been safely given to PD patients and has known symptomatic benefits similar to selegiline.[51] While selegiline has been widely studied, there is still uncertainty about putative neuroprotective and symptomatic effects (see Appendix 1). On balance, there is sufficient suggestive evidence of a neuroprotective effect for this class of agents to warrant additional, definitive clinical trials.

Ropinirole and pramipexole. These are nonergot derivative dopamine agonists. There are several suspected mechanisms including antioxidant action and direct effects on the mitochondrial membrane.[52] These effects may be a more broad property of other dopamine agonists as well. There is abundant evidence showing that early use of dopamine agonists rather than levodopa reduces the incidence of motor complications. In one such study, pramipexole-treated patients showed a reduced rate of dopamine transporter loss as measured by SPECT with 2β-carboxymethoxy-3β(4-iodophenyl)tropane (β-CIT) labeled with iodine 123[53]. The exact interpretation and clinical meaning of these data remain unclear.[54,55]

Discussion. We attempted to systematically identify and assess potential compounds for neuroprotection clinical trials in PD. Fifty-nine compounds were identified by clinicians and scientists from academics, government, and industry. Twelve were judged to be worthy of further study for neuroprotection clinical trials in the near term. These agents reflect a variety of mechanisms consistent with current theories about disease pathogenesis. These agents are in different stages of development. For example, drugs such as caffeine or nicotine would clearly require more preliminary data on dosing and administration before they could be considered for comparative efficacy studies. It should be noted that this is not an exhaustive list of potential neuroprotective agents; there are clearly promising agents in earlier stages of development, interventions requiring surgery, and

proprietary agents not discussed here. However, the results of this review show that there are compounds already available that should be pursued aggressively in trials aimed at slowing the progression of PD.

While we attempted to make the criteria explicit, qualitative judgments had to be made about the relative value and weighting of different types of information. The most problematic issue was the evaluation of animal data. Preclinical models of parkinsonism are currently of uncertain value in predicting results in humans. There are several rodent models, including lesion models such as MPTP, 6-OHDA, metamphetamine, chronic rotenone administration, and more recently, transgenic synuclein models. Additionally there are nonhuman primate lesion models, as well as aged primate models. There are also models of other diseases, such as the SOD knock-out mouse, that may be relevant to PD. The diversity of animal models creates a practical problem in terms of comparability of preclinical efficacy data, both pathologic and behavioral, as well as the time and resources to review all preclinical data for multiple drugs. However, given the multiple mechanisms that appear to be involved in the pathogenesis of PD, drugs that offer protection in more than one model may be particularly attractive to study in clinical trials.

Each preclinical model has different advantages for research. While lesion models like MPTP clearly do not recapitulate the degenerative process in PD, they have been useful in modeling aspects of parkinsonism such as dyskinesias and in the development of dopaminergic drugs. MPTP and 6-OHDA are currently the most widely used, and the vast majority of drugs considered for PD have been tested in these models. Newer transgenic models are emerging that may model the degenerative process better, but there are differences in their clinical phenotype that must be addressed and standardized. At the time of this writing there were no published results of animal trials using transgenic PD models. The predictive validity of all these PD models for human neuroprotection trials is unknown, and a positive trial is needed to show predictive validity of any preclinical model. Upcoming trials are an opportunity to test and compare both older lesion models and newer transgenic models for their predictive value in humans. It is clear, however, that there is a pressing need to further develop preclinical models of PD.

Many of the drugs discussed here have well-known, potentially confounding symptomatic effects that may be considered obstacles in planning future clinical trials aimed at demonstrating neuroprotection. Trial design remains a complicated and challenging issue in the field of neuroprotection, and there are no simple solutions. Conceptually there are a limited number of ways to deal with symptomatic effects of the drug in the trial design. One commonly used approach is to focus on the interpretation of the primary clinical outcome. Additional measures such as neuroimaging or other biomarkers may assist in the interpretation of the main clinical findings. This approach has been used in recent trials of dopamine agonists, but it is clear that imaging markers may also be susceptible to confounding and modulation and a better understanding of these markers is needed before imaging can be used to interpret clinical findings.[55] Another approach is to account for symptomatic effects in the design by using a delayed start, or a wash-in, of the placebo group at the end of the study. This may be preferable to a wash-out period, which is subject to uncertainty about the adequacy of the withdrawal period. A third approach is to forgo the problematic clinical distinction between protective and symptomatic therapies. Agents are selected based on their potential to slow disease progression, but to be clinically useful a treatment must be shown to make patients' symptoms better than the current standard of care. In this view, neuroprotective therapies should address unmet therapeutic needs such as postural instability and cognitive impairment and should prolong the years during which PD patients experience well-controlled motor symptoms. There are clearly obstacles to each of these approaches. However, concerns about the symptomatic effects of a drug should not prohibit its testing in trials designed to demonstrate sustained benefits in PD patients, although the mechanism underlying such benefits could remain unclear.

Many of the agents described here are well known to clinicians and researchers. While they are familiar, they have not been adequately explored for the purposes of neuroprotection in PD. In fact, few interventional strategies have been tested in clinical trials designed or powered to detect clinically meaningful differences in disease progression. Most neuroprotection trials have studied the MAO-B inhibitor selegiline, although uncertainty remains about the role of this drug in PD. Trials of dopamine agonists have yielded controversial neuroimaging results and conflicting results on the endpoints of motor complications and other measures of motor function.

The approach used here to evaluate existing drugs for their use in PD is explicit, systematic, and transparent. It is intended to facilitate public scrutiny of resource-intense clinical trials and provides a framework for researchers to prioritize interventions. More candidate drugs will be identified as academic researchers adopt high throughput screening techniques and study candidate neuroprotective drugs in new genetic models of PD. Thus, the need to prioritize will increase. This approach to systematically evaluating drugs could be extended to be more inclusive, but the value of such a database, in the long term, would depend on the broad participation of the PD research community.

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A SYNDROME OF SEIZURES AND PERVASIVE DEVELOPMENTAL DISORDER ASSOCIATED WITH EXCESSIVE CELLULAR NUCLEOTIDASE ACTIVITY

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INTRODUCTION

Pervasive Developmental Disorder (PDD) is defined broadly as impaired reciprocal social interaction, communication, and imaginative activity combined with a markedly restricted repertoire of activities and interests.[1] PDD includes such disorders as childhood schizophrenia, infantile autism, and Asperger's syndrome. PDD is often associated with neurological symptoms such as seizures and ataxia. A number of single-gene defects and chromosomal abnormalities are associated with PDD; these include Fragile X, Down's syndrome, histidenemia, phenylketonuria, neurolipidosis, and tuberous sclerosis.

We report here a unique type PDD which is associated with seizures and other neurological symptoms, abnormal speech and behavior, and increased susceptibility to infection. All of these patients show excessive 5′ nucleotidase activity in their fibroblast lysates. Metabolic therapy with oral uridine brings about dramatic improvement in every case.

CLINICAL PRESENTATION

The clinical presentation of four patients is summarized in Table 1, and was fairly consistent. All patients were markedly delayed in their developmental milestones, especially language. All had seizures, ataxia, an awkward gait, and mildly impaired fine motor control. All four displayed an unusual behavioral phenotype which was characterized by extreme hyperactivity, distractability, a strange "delirious" quality to their affect, and abnormal social interaction. All four patients experienced frequent ear and sinus infections but no consistent reason for immunodeficiency (such as reduced antibody titre or abnormal T-cell

Purine and Pyrimidine Metabolism in Man IX,
edited by Griesmacher *et al.* Plenum Press, New York, 1998.

Table 1. Clinical presentation of four patients

	Symptom	Patient 1	2	3	4
General	sex	F	F	M	M
	age first studied (years)	3	4	2	8
	growth retardation	+	–	+	+
Behavior	hyperactive	+	+	+	+
	inability to focus	+	+	+	+
	extreme distractability	+	+	+	+
	occasionally aggressive	+	–	+	+
	impulsive	+	+	+	+
	"delirious" affect	+	+	+	+
	compulsiveness	+	+	+	+
	abnormal social interaction	+	+	+	+
Speech	speech delay	+	+	+	+
	slurred speech	+	+	+	+
	tremulous speech	+	+	+	+
	short, telegraphic sentences	+	+	+	+
Neurological	seizures	+	+	+	+
	abnormal EEG	+	+	+	+
	ataxia	+	+	+	+
	impaired fine motor control	+	+	+	+
	awkward gait	+	+	+	+
Immunological	frequent infections	+	+	+	+
	abnormal immunoglobulins	+	+	–	–
	abnormal T-cell response	+	+	–	–
Other	developmentally delayed	+	+	+	+
	sparse hair/ hair loss	+	+	–	+
	skin rash	+	+	+	–
	urate excretion mg/mg creatinine	0.76	0.50	0.41	0.45

response) could be found. All patients excreted reduced quantities of uric acid when compared with age-matched controls[2], but other metabolic tests, such as plasma and urinary amino acids and organic acids were found to be within normal limits.

MATERIALS AND METHODS

Incorporation of purine and pyrimidine precursors into nucleotides in intact cultured fibroblasts was done as previously described[3]. Concentrations of intracellular nucleotides were measured by harvesting 4×10^6 cultured skin fibroblasts in the log phase of growth by trypsinization, extraction of the nucleotides by 100 ul of 0.5 M perchloric acid, neutralization with potassium phosphate buffer, and analysis by HPLC. Individual enzymes were assayed in fibroblast lysates by previously described methods.[4]

RESULTS AND DISCUSSION

Studies of the incorporation of purine and pyrimidine precursors into nucleotides as well as individual enzyme assays were performed to determine the basis of the observed

Table 2. Incorporation of precursors into nucleotides

Precursor	Pt 1	Pt 2	Pt 3	Pt 4	Controls (n)
Adenine	9472	9792	8993	9025	9170 (6)
Hypoxanthine	3246	3686	3071	3387	3043 (6)
Guanine	3429	2970	2893	2913	3242 (6)
Formate	5046	6681	6269	5890	7842 (3)
Uridine	3982	5840	4876	6137	8655 (3)

Incorporation of formate into nucleotides is in units of pmol/ 100 nmol UV/24 hr; all others are in units of pmol/100 nmol UV/2 hr

reduced uric acid excretion. The results of the incorporation studies are shown in Table 2. Incorporation of adenine, guanine, and hypoxanthine into nucleotides appears to be normal. Incorporation of formate into nucleotides, a measure of *de novo* purine synthesis, was found to be slightly reduced. Incorporation of uridine into pyrimidine nucleotides was also reduced. In all of the incorporation studies, no abnormal distribution of labeled nucleotides which would suggest a deficiency of any of the enzymes of nucleotide interconversion was seen.

Individual enzymes of pyrimidine metabolism which might affect the incorporation of uridine were assayed in fibroblast lysates. As seen in Table 3, all of these enzyme activities were normal with the exception of 5′ nucleotidase, which was consistently 6- to 8-fold greater in the patients compared to age-matched controls. This increased nucleotidase activity was seen with purine substrates, such as AMP, as well.

To determine whether the increased nucleotidase acitivity caused any abnormality in steady-state nucleotide concentrations, nucleotide concentrations were measured in fibroblasts. All nucleotide levels appeared to be within normal limits (Table 4).

With consideration to the possibility that pyrimidine nucleotide deficiency in some cell type might be responsible for these symptoms, pyrimidine replacement therapy was initiated with uridine at a dose of 1000 mg/kg/day.

Table 3. Enzyme activities in fibroblast lysates

Enzyme (substrate)	Pt 1	Pt 2	Pt 3	Pt 4	Controls (n)
5′ Nucleotidase (UMP)	7.48	8.21	7.61	8.67	1.14 (9)
5′ Nucleotidase (AMP)	7.94	9.54	8.87	9.41	1.54 (6)
UMP Synthetase (Orotate)	2.55	1.56	1.95	2.27	1.99 (5)
Uridine Kinase (uridine)	3.41	3.09	4.06	3.53	3.85 (3)
Nucleoside Phosphorylase (Uridine)	0.59	0.39	0.65	0.44	0.48 (5)

Enzyme activities are in units of nmol/min/mg protein.

Table 4. Nucleotide concentrations in cultured fibroblasts

Nucleotide	Pt 1	Pt 2	Pt 3	Pt 4	Controls (5)
ATP	11.1	12.2	10.7	11.5	13.7
ADP	3.4	2.5	3.7	3.1	3.4
GTP	3.8	4.7	4.3	4.3	4.9
GDP	0.5	0.3	0.2	0.3	0.5
UTP	3.2	3.7	4.1	4.5	4.1
CTP	0.8	0.8	1.1	0.8	1.0

Nucleotide concentrations are in units of nmol/10^6 cells.

At this dose seizure activity was eliminated or greatly reduced, and other neurological symptoms improved as well. Speech became more normal and age-appropriate. Improvements in behavior and social interaction were also noted, and infections became less frequent. As the patients seemed to derive considerable benefit from uridine, a double-blind crossover study was undertaken to test the efficacy of uridine. The switch to placebo was accompanied by regression to the pretreatment state in virtually all areas. Performance on standardized tests of cognitive function also deteriorated while on placebo.

The metabolic basis of these symptoms is presently unknown. There is no evidence of pyrimidine nucleotide deficiency in the cells of these patients, and the disorder bears little resemblance to orotic aciduria. The rapid and impressive response to uridine and the rapid regression when uridine is discontinued argues against a neurodevelopmental defect. Rather, the increased nucleotidase activity appears to produce a continual deficiency of some essential metabolite, and this deficiency is corrected by uridine.

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A SYNDROME OF MEGALOBLASTIC ANEMIA, IMMUNODEFICIENCY, AND EXCESSIVE NUCLEOTIDE DEGRADATION

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INTRODUCTION

Several defects of purine and pyrimidine metabolism have been associated with behavioral abnormalities. The most common and best studied is Lesch-Nyhan syndrome[1], with its characteristic aggressive, self-mutilating behavior. Deficiency of adenylosuccinate lyase[2] has been reported to be associated with infantile autistic behavior. Autistic behavior as well as seizures have been associated with thymine-uraciluria[3]. We report here a syndrome which involves behavioral abnormalities, seizures, and macrocytic anemia and which is associated with increased degradation of purine and pyrimidine nucleotides.

CASE HISTORY

The patient, a three-year-old white female, was first seen because of recurrent infections, developmental delay, and seizures. Upon examination, she was found to have mild immunodeficiency, macroycytic anemia, ataxia, and alopecia. Physical development and speech were notably delayed. IgG was borderline to low, and MCV was variable from 90 to 100 (normal for age and sex <88). A severe, recurrent sinus infection required surgical drainage. No abnormalities of amino acid or organic acid metabolism were identified by a routine metabolic screen and amino acid analysis. No unusual compounds were detected in plasma or urine by HPLC. All parameters of folic acid and B12 metabolism were found to be within normal limits. The most striking feature of her phenotype was her bizarre, and often aggressive behavior. She was hyperactive, with a short attention span, inappropriate verbalizations, and poor interaction with other children. Aggressive behavior took the form of pinching or scratching others, or biting toys. She would sometimes bang her head or poke at her eye with her finger. She had 2-3 seizures of 1-2 min duration per day. Initially, she was treated with IgG, folinic acid, depakote, and tegritol. Her sinus infection resolved and seizure activity decreased but there was no change in her MCV, behavior, or speech development. At this time, an investiga-

Purine and Pyrimidine Metabolism in Man VII, Part B
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tion of her nucleotide metabolism in cultured fibroblasts was begun. Based on the findings of these studies, a trial with oral nucleotides was begun. Upon initiation of this treatment, an almost immediate improvement in speech, behavior, and cognitive function was seen. Speech became more understandable and appropriate and she seemed to pay more attention to her surroundings, and focus better on tasks. Her interactions and play with other children became appropriate, and her mother described her behavior as that of a normal child. MCV remained elevated. Seizure activity decreased markedly, such that she was taken off depakote (625 mg/day), and the dose of tegritol was gradually reduced from 500 to 50 mg/day, with the intention of eliminating it as well. However, an interuption in the supply of nucleotides caused a one week interuption in oral nucleotide therapy. During this time, seizure activity increased to >10 seizures per day. Her attention span became limited, and her frustration tolerance low. Verbalization and interaction with others deteriorated, and behavior became more aggressive. At that time she was returned to pretreatment doses of depakote and tegritol. Upon resumption of nucleotide therapy, these symptoms resided, and her condition prior to the interuption of therapy gradually returned.

MATERIALS AND METHODS

Incorporation studies were done as described earlier[4] for adenine, guanine, hypoxanthine, formate, uridine, and thymidine. For glycine and orotic acid incorporation studies, isotope (10 uCi/ml) was added to Minimal Essential Medium and cells were grown in 75 mm plates for 72 hr, harvested by trypsinization, and analyzed by HPLC as above. Incorporation experiments were done in duplicate. For the assay of individual enzymes, cultured fibroblasts were harvested in the log phase of growth and lysed at a concentration of approximately 1 mg/ml in a 0.10 sodium phosphate buffer, pH 7.2 containing 0.05 M magnesium chloride. Serial dilutions of this lysate were incubated with a 10 uM concentration of radiolabeled substrate for 1 hr at 37^{0}C. For the assay of UMP synthetase 1 mM PRPP was added. For the assay of uridine kinase, 1 mM ATP was added. The assays were deproteinized and analyzed by HPLC. Assays were done in triplicate. Reported values do not necessarily represent maximum enzyme activities due to the low substrate concentration. Nucleoside inhibition of erythroid colony formation by bone marrow cells was done as described earlier[5].

RESULTS AND DISCUSSION

The incorporation of purine and pyrimidine precursors into nucleotides is shown in Table 1. Normal incorporation of glycine into purines, as well

Table 1. Incorporation of Precursors into Nucleotides

Precursor	Patient	Controls (n)
adenine	8160	9727 (4)
hypoxanthine	3308	2849 (4)
guanine	3392	3129 (4)
formate + AICAR	3688	3658 (4)
glycine	10071	8350 (2)
uridine	3469	8511 (4)
thymidine	1223	1027 (2)
orotic acid	5694	18315 (2)

Incorporation is reported in units of pmol/100 nmol purines/2 hr

as normal excretion of uric acid by the patient indicate normal denovo purine synthesis. From the incorporation of adenine, hypoxanthine, and guanine into the various purine nucleotides it is clear that the activities of enzymes of purine nucleotide interconversion (i.e. adenylosuccinate synthetase, adenylosuccinate lyase, AMP deaminase, IMP dehydrogenase GMP, synthetase, GMP reductase, and the purine nucleotide mono- and diphosphate kinases) are comparable to those of normal controls (data not shown). Similarly, the production of normal amounts of UTP, CTP, and TTP from uridine indicates that the enzymes of pyrimidine nucleotide interconversion (i.e. CTP synthetase, thymidylate synthetase, and the pyrimidine mono- and diphosphate kinases) are comparable to normal controls. The only notable differences in these precursor incorporation studies was the low incorporation of orotic acid and uridine into pyrimidine nucleotides. This could reflect low activities of the synthetic enzymes or increased catabolism of the nucleotide products.

To study this question further, individual enzyme activities in dialyzed fibroblast lysates were measured (Table 2). To determine if a deficiency existed in the synthesis of pyrimidine nucleotides, the activities of UMP synthetase and uridine kinase were measured, and found to be normal. The only consistent difference between the patient and normal controls was a ten- to thirty-fold increase in the catabolism of UMP. When the rate of purine nucleotide catabolism was measured for comparison a similar elevation was found. Interestingly, the increased catabolism of purine nucleotides had no noticeable effect on the incorporation of purine precursors into purine nucleotides, whereas the increased catabolism of pyrimidine nucleotides appeared to result in a net decrease in pyrimidine nucleotide synthesis, as measured in cultured fibroblasts. To further study the metabolism of pyrimidine nucleotides in intact cells, the effect of pyrimidine nucleosides on erythroid colony formation was measured in the presence and absence of nucleosides (Table 3). Clearly, the patient's bone marrow cells show much less inhibition of colony formation in presence of thymidine and uridine than control bone marrow cells. This indicates that in the patient's cells, pyrimidine nucleosides are either transported or phosphorylated more slowly, or that the pyrimidine nucleotides, once formed, are degraded more rapidly. Again, it is interesting to note that this effect is present with thymidine, although intact fibroblasts show no decrease in the incorporation of thymidine into thymidine nucleotides.

On the basis of these results, it was decided to intiate pyrimidine nucleotide replacement therapy. In orotic aciduria, in which there is a known defect in pyrimidine synthesis associated with macrocytic anemia, pyrimidine nucleotide replacement therapy has been quite successful. The patient was started with 150 mg/kg/day each of UMP and CMP Plasma uri-

Table 2. Activities of Catabolic Enzymes in Dialyzed Cell Lysates

Enzyme (substrate)	Patient	Controls (n)
5'Nucleotidase (UMP)	7.44	0.65 (4)
5'Nucleotidase (AMP)	9.64	0.80 (4)
Nucleoside Phosphorylase (uridine)	0.41	0.44 (4)
Nucleoside Phosphorylase (inosine)	5.72	4.24 (4)
Adenosine Deaminase	3.54	5.09 (4)
UMP Synthetase	1.56	2.17 (4)
Uridine Kinase	2.86	3.51 (2)

Enzyme activities are in nmol/min/mg protein

Table 3. Inhibition of Erythroid Colony Formation by Nucleosides

Nucleoside (concentration)	Patient	Control
None	347 (100%)	169 (100%)
Uridine (10 uM)	322 (94%)	166 (98%)
Uridine (50 uM)	320 (94%)	164 (97%)
Uridine (75 uM)	335 (98%)	56 (33%)
None	508 (100%)	328 (100%)
Thymidine (10 uM)	486 (95%)	251 (76%)
Thymidine (50 uM)	298 (59%)	0 (0%)
Thymidine (75 uM)	96 (18%)	0 (0%)

Colony formation in units of average colonies per plate

dine and erythrocyte UTP were monitored during therapy. Plasma uridine was undetectable before therapy and stayed in the range of 20-50 uM during therapy. Erythrocyte UTP was similarly undetectable before therapy, and was maintained in the range of 20-60 nmol/ml packed red cell during therapy. A general improvement in the patient's condition was noted, but MCV remained abnormally high. Because increased catabolism of purine nucleotides was also indicated, 75 mg/kg/day AMP was included, and the daily dose of pyrimidines was increased to 500 mg/kg/day. These measures produced no additional improvement, and MCV remained high.

At present, the precise metabolic basis of these symptoms remains unknown. The increase in nucleotidase activity could be the primary defect, or it could be a response to abnormal amounts of some as yet unidentified nucleotide. The fact that MCV remained high, even during nucleotide replacement therapy with adenine, cytidine, and uridine nucleotides might indicate a shortage of other nucleotides, perhaps deoxynucleotides. Alternatively, the amount of nucleotides used here may have been inadequate to maintain normal nucleotide levels in the presence of increased nucleotidase activity.

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